(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 November 2002 (28.11.2002)

PCT

(10) International Publication Number
WO 02/094880 A1

(51) International Patent Classification[7]: C07K 16/28, C12N 5/10, C12P 21/08, C12N 15/13, 1/21, 1/19, A61K 39/395

(21) International Application Number: PCT/JP02/04816

(22) International Filing Date: 17 May 2002 (17.05.2002)

(25) Filing Language: Japanese

(26) Publication Language: Japanese

(30) Priority Data:

2001-150213	18 May 2001 (18.05.2001)	JP
2001-243040	9 August 2001 (09.08.2001)	JP
2001-314489	11 October 2001 (11.10.2001)	JP

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
- *with international search report*

For two-letter codes and other abbreviations, refer to the Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ANTI-TRAIL-R ANTIBODIES

(57) Abstract: Antibodies against TRAIL-R1 and TRAIL-R2 having at least one characteristic selected from among the following (a) to (c) or functional fragments thereof: (a) having an activity of inducing apoptosis of cancer cells expressing TRAIL-R1 and/or TRAIL-R2; (b) exerting no effect on normal human cells expressing TRAIL-R1 and/or TRAIL-R2; and (c) inducing no injury on human hepatocytes.

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SPECIFICATION

ANTI-TRAIL-R ANTIBODIES

TECHNICAL FIELD

The present invention relates to anti-TRAIL receptors (TRAIL-R) which identify TRAIL receptor 1 (TRAIL-R1) or TRAIL receptor 2 (TRAIL-R2) which are molecules in the cell membrane involved in apoptosis.

Furthermore, the present invention relates to agents for treating or preventing diseases caused by cells expressing TRAIL-R, particularly to agents for treating malignant tumors, having anti-TRAIL-R antibodies as the active ingredient.

BACKGROUND ART

The biological cell death which occurs for the purpose of healthy cell replacement *in vivo* is called apoptosis, as distinguished from necrosis, which refers to pathological cell death [see Kerr *et al.*, (1972), *Br. J. Cancer* 26, 239]. Apoptosis is a phenomenon which can be generally observed in the process of embryogenesis and lymphocyte (T cells and B cells) selection [see Itoh, S. *et al.*, (1991), *Cell* 66, 233-243]. The failure of cells which should be eliminated to actually be eliminated is believed to be a possible cause of cancer, lupus, herpes infections and the like. Additionally, if cells which should be alive are eliminated by apoptosis, this can cause such diseases and disorders as AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, aplastic anemia, myocardial infarction, cerebral apoplexy and hepatopathy due to toxic substances [see Kataoka, S. *et al.*, (1996) *The Oncologist* 1, 399-401].

During apoptosis, such phenomena as curvature of the cell surface, condensation of nucleic chromatin, fragmentation of chromosomal DNA and failure of mitochondrial functions are characteristically observed. These types of cell changes are believed to be caused by various intrinsic and extrinsic signals. As for intrinsic signals, oncogenes such as *myc* and *bcl*-2 and anti-oncogenes such as p53 have been reported to be involved in inducing apoptosis [see Kataoka, Shiro *et al.* (1993) *Jikken Igaku* 11, 17, 2324-2328]. As for extrinsic signals, it is known that chemotherapeutic agents and radiation can induce apoptosis [Kataoka, Shiro *et al.* (1994) *Saishin Igaku* 49, 6, 1152-1157].

Molecules belonging to the tumor necrosis factor family (TNF family) such as tumor

necrosis factor α (TNF-α), tumor necrosis factor β (TNF-β) and Fas ligands have been identified as being molecules involved in apoptosis. TNF-α and TNF-β have been reported to indue apoptosis in cancer cells [see Schmid *et al.* (1986) *Proc. Natl. Acad. Sci.* 83, 1881; Dealtry *et al.* (1987) *Eur. J. Immunol.* 17, 689]. Since mutant murine Fas or Fas ligands indicate the condition of an autoimmune disease, this strongly suggests that Fas ligands have the task of eliminating peripheral auto-antigen reactive lymphocytes [see Krammer *et al.* (1994) *Curr. Op. Immunol.* 6, 279-289; Nagata *et al.* (1995) *Science* 267, 1449-1456]. Agonistic murine monoclonal antibodies specifically binding to Fas have been reported to exhibit the same level of apoptosis-inducing activity as TNF-α with respect to cancer cells [see Yonehara *et al.* (1989) *J. Exp. Med.* 169. 1747-1756].

These TNF family molecules transmit signals into cells by binding with specific receptors on the cell surface. Numerous receptors for TNF family molecules are known, and these are called TNF receptor family molecules.

While molecules in the TNF receptor family are defined by the presence of cysteine-rich repetitions in the extracellular domain, Fas and TNFR1, which are the receptors for Fas ligands and TNF-α, have an intracellular domain which is necessary for the transmission of the apoptosis signal, a domain known as the "death domain" which is homologous to the Drosophila suicide gene *reaper* [see Golstein, P. *et al.* (1995) *Cell* 81, 185-186; White, K. *et al.* (1994) *Science* 264, 677-683]. Activation of Fas prompts association with the adaptor molecule FADD/MORT1 containing the death domain, thereby causing activation of caspase-8 which is bound to the FADD/MORT1. Once the caspase-8 is activated, the entire group of caspase molecules downstream thereof is activated in sequence, ultimately leading to cell apoptosis [see Nagata S. (1997) *Cell* 88, 355-365].

Recently, a TNF family molecule which induces apoptosis has been newly discovered. Wiley *et al.* [see *Immunity* (1995) 3, 673-682] named the molecule "TNF-related apoptosis-inducing ligand", or simply "TRAIL". This molecule is also known as "Apo-2 ligand" or "Apo-2L" [see Pitt, R. M. *et al.* (1996) *J. Biol. Chem.* 271, 12687-12690]. For convenience's sake, this molecule shall be referred to in this specification as "TRAIL".

Unlike Fas ligands, significant levels of TRAIL are detected in the tissues of most humans (for example, the spleen, lungs, prostate, thymus, ovaries, small intestine, large intestine, peripheral blood lymphocytes, placenta and kidneys). Additionally, it is constantly transcribed in a number of cell lines. TRAIL has also been demonstrated to activate apoptosis

very quickly, even faster than TNF-induced apoptosis, in a time frame similar to the transmission of death signals by Fas [see Marsters, S. A. *et al.* (1996) *Curr. Biol.* 6, 750-752].

At present, five proteins have been identified as being TRAIL receptors. The two receptors TRAIL-R1 (also called DR4) and TRAIL-R2 (also called DR5) both have been reported to have death domains in the intracellular domain. The transcription products of TRAIL-R1 have been observed in many types of human tissue, such as in the spleen, peripheral blood leukocytes, small intestine and thymus. The transcription products of TRAIL-R2 have also been observed in many types of tissue, including the spleen, peripheral blood lymphocytes and ovaries [see Pan, G. *et al.* (1997) *Science* 276, 111-113; Pan, G. *et al.* (1997) *Science* 277, 815-818; Walczak, H *et al.* (1997) *EMBO J* 16 (17) 5386-397].

TRAIL-R2 has two possible forms due to alternative splicing, and in cancer cells, a high rate of expression has been reported for the TRAIL-R2 composed of 440 amino acids [see Screaton, G. R. *et al.* (1997) *Curr Biol* 7 (9), 693-696; Arai, T *et al.* (1998) *Cancer Letters* 133, 197-204].

Recombinant human TRAIL is a recombinant protein composed of the extracellular domain of TRAIL, which has been reported to induce apoptosis in numerous types of cancer cells [see Griffith, T. S. *et al.* (1998) *Curr. Opin. Immunol.* 10, 559-563].

Furthermore, recombinant TRAIL has been effective in a cancerous mouse model using human colon cancer cells and breast cancer cells [see Walczak, H *et al.* (1999) *Nature Medicine* 5, 2, 157-163]. Unlike TNF-α and Fas ligands which also belong to the TNF receptor family and have apoptosis-inducing activity, TRAIL did not damage healthy tissue in mice or cynomolgus mokeys [see Ashkenazi, A. *et al.* (1999) *J. Clin. Invest.* 104, 155-162].

While these reports led to the conjecture that TRAIL induces cell death selectively in tumors, TRAIL receptors are also expressed in healthy cells and there has not yet been any theoretical support for such selectivity. Furthermore, a recent report has shown that recombinant human TRAIL induces apoptosis in healthy human hepatocytes [see Jo, M. *et al.* (2000) *Nature Medicine* 6, No. 5, 564-567]. Additionally, it has also been reported to induce apoptosis in human brain cells [see Nitsch, R *et al.* (2000) *The Lancet* 356, 827-828]. The fact that agonistic anti-Fas antibodies which induce apoptosis in hepatocytes can bring about fulminant hepatitis and cause death in mice and chimpanzees in an extremely short time has forced a reconsideration of the possibility of cell death induced in hepatocytes by TRAIL, with questions being raised concerning the safety of TRAIL if used as a pharmaceutical in humans [see Nagata,

S. (2000) *Nature Medicine* 6, 5, 502-503].

While it has been reported that the ability of TRAIL to induce cell death in hepatocytes depends on the type of recombinant TRAIL protein [see Lawrence, D. *et al.* (2001) *Nature Medicine* 7, 4, 383-385], the question of the safety of recombinant TRAIL proteins is still in the research stage.

Recently, an anti-Fas antibody which did not cause liver damage when administered to mice was reported for the first time [see Ichikawa, K. *et al.* (2000) *International Immunology* 12, NO. 4, 555-562]. No recombinant Fas ligands are known to have been confirmed not to cause liver damage. This suggests that it is possible to obtain an antibody having activity which cannot be expected in ligands. However, the reasons that these antibodies do not exhibit hepatotoxicity despite inducing apoptosis in T-cells have yet to be clarified in theoretical form, and it is still unclear whether non-toxic agonistic antigens can be obtained in the case where the antigens differ such as with TRAIL.

While TRAIL induces apoptosis by acting on TRAIL-R1, TRAIL-R2 or both, it is not yet clear which receptor is used by the signal when TRAIL induces apoptosis in hepatocytes. Moreover, there has been no research concerning whether or not hepatotoxicity can be avoided by incorporating TRAIL-R1/R2 selectivity in agonistic antibodies.

An effective means for treating malignant tumors is to remove the cancer cells and protect the healthy tissue or cells. Although medications making use of apoptosis induction by recombinant human TRAIL as the mechanism of action may be effective for eliminating cancer cells, they may also cause damage to healthy tissue, particularly the liver and brain.

Currently, monoclonal antibodies such as humanized antibodies targeting Her2/neu and chimeric antibodies targeting CD20 which are receptors present in the cell membrane are used against malignant tumors, and their therapeutic effects have been confirmed. Antibodies are characterized by a long half-life in the blood and a high specificity to antigens, and are particularly useful as anti-tumor agents. For example, if the antibodies target tumor-specific antigens, then the administered antibodies can be expected to gravitate toward the tumor, due to which the immune system can be expected to attack the cancer cells by complement-dependent cell damage and antibody-dependent cell damage. Additionally, by binding such agents as radionuclides and cytotoxic substances to the antibodies, the agents bound in this way can be efficiently delivered to the tumor portion, while simultaneously reducing the amount of the agent delivered to non-specific tissue, thus resulting in decreased side effects. If the

tumor-specific antigens have activity which induces cell death, then an antibody having agonistic activity can be administered, and if the tumor-specific antigens are involved in the proliferation and survival of cells, then an antibody having neutralizing activity can be administered, which can be expected to result in inhibition on proliferation and regression of the tumor due to the accumulation of tumor-specific antibodies and their activity.

Due to the above-described characteristics, antibodies are believed to be appropriate for application as an anti-tumor agent. Furthermore, if the antibodies are active against TRAIL receptors, it may be possible to obtain one that can avoid damage to the liver which cannot be avoided with recombinant human TRAIL itself, and has the same level of apoptosis-inducing activity against cancer cells.

DISCLOSURE OF THE INVENTION

A first object of the present invention is to offer a novel antibody or similar molecule which can bind to human TRAIL-R1 and/or human TRAIL-R2, induces apoptosis specifically with respect to cancer cells, and does not cause damage to healthy human hepatocytes which can be damaged by recombinant human TRAIL proteins. The second object is to offer an agent for preventing or treating various types of malignant tumors, such as solid tumors which are currently difficult to treat, comprising the aforementioned antibody or similar molecule as the active ingredient.

As a result of diligent research toward the production of an antibody against human TRAIL-R1 and R2, in which the present inventors immunized transgenic mice having the ability to produce human antibodies due to genetic engineering techniques with human TRAIL-R1 and R2, and used the method of Kohler and Millstein [see (1975) *Nature* 256, 495] which is commonly used to produce monoclonal antibodies, they succeeded in making a hybridoma producing a novel monoclonal antibody binding to TRAIL-R1 and TRAIL-R2, and obtaining the monoclonal antibody from the conditioned medium. They then discovered that this novel monoclonal antibody can bind to TRAIL-R1 and/or R2 on the surfaces of cancer cells to induce apoptosis specifically in cancer cells, thereby completing the present invention.

That is, the present invention can be defined as follows.

(1) An antibody or functional fragment thereof binding to TRAIL-R1 and/or TRAIL-R2.

The above-described antibody and functional fragment thereof have at least one

selected from among the following properties (a)-(c):

(a) having activity for inducing apoptosis in cancer cells expressing TRAIL-R1 and/or TRAIL-R2;

(b) not affecting healthy human cells expressing TRAIL-R1 and/or TRAIL-R2; and

(c) not causing damage to human hepatocytes.

In the present invention, an antibody or functional fragment thereof having all of the above-described properties (a)-(c) is preferable. Additionally, the antibodies and functional fragments thereof according to the present invention include antibodies or functional fragments thereof having at least one of the above-described properties (a)-(c), and (1) binding to TRAIL-R2 but not binding to TRAIL-R1, or (2) binding to both TRAIL-R1 and TRAIL-R2.

(2) The above-described antibodies are monoclonal antibodies, preferably human antibodies, produced by mouse-mouse hybridomas such as E-11-13, H-48-2, L-30-10, N-18-12, W-40-5, X-14-4, X-51-12, F-4-8, G-3-10, 0304 or KMTR1. The type of monoclonal antibody produced by -11-13, H-48-2, L-30-10, N-18-12, W-40-5, X-14-4, X-51-12, F-4-8, G-3-10, 0304 or KMTR1 is immunoglobulin G (IgG), while the type of monoclonal antibody produced by G-3-10 is immunoglobulin M (IgM). Here, of the above-described hybridomas, H-48-2, E-22-13, F-4-8, L-30-10, 0304 and KMTR1 have all been internationally deposited, their deposit information being as follows.

Name	Deposit No.	Deposit Date	Deposit Location
H-48-2	FERM BP-7599	May 18, 2001	International Patent Organism Depositary at the National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan)
E-11-13	FERM BP-7698 FERM BP-7770	August 8, 2001 October 11, 2001	
F-4-8	FERM BP-7699 FERM BP-7768	August 8, 2001 October 11, 2001	
L-30-10	FERM BP-7700 FERM BP-7769	August 8, 2001 October 11, 2001	
0304	FERM BP-8037	May 10, 2002	
KMTR1	FERM BP-8038	May 10, 2002	

Cancer cells include colon cancer cells Colo205, U251 cells from neuroglioma or Jurkat cells from T-cell lymphoma, and are chosen as appropriate from among these cells.

(3) The antibody or functional fragment thereof according to the present invention has an LD50 value with respect to human hepatocytes, under conditions of 7.5×10^4 cells and a reaction time of 24 hours, of at least 0.01 μg/ml, preferably at least 0.1μg/ml, more preferably 2-10μg/ml, even more preferably 10-100μg/ml, and most preferably at least 10μg/ml (for example,

100μg/ml or more). On the other hand, the antibody or functional fragment thereof according to the present invention has an LD50 value with respect to cancer cells (for example, Colo205 cells, U251 cells or Jurkat cells), under conditions of 2.5×10^3 cells and a reaction time of 48 hours, of at most 100 μg/ml, preferably at most 10 μg/ml, more preferably at most 0.7 μg/ml, even more preferably 0.02-0.11 μg/ml, and most preferably at most 0.02 μg/ml. Additionally, an antibody or functional fragment thereof of a combination such as to have an LD50 value of 2-100 μg/ml with respect to human hepatocytes at 7.5×10^4 cells and a reaction time of 24 hours, and an LD50 value of 0.02-0.11 μg/ml with respect to cancer cells at 2.5×10^3 and a reaction time of 48 hours is especially preferable for the present invention.

Here, the above-described LD50 of the antibody of the present invention with respect to hepatocytes or cancer cells is a value as measured at a reaction volume of 110-120 μl per reaction system (per well).

(4) Additionally, the antibody or functional fragment thereof according to the present invention is such that the LD50 value with respect to human hepatocytes at 7.5×10^4 cells and a reaction time of 24 hours is at least twice, preferably at least 10 times, more preferably at least 50 times (for example, 50-100 times), even more preferably at least 100 times (for example, 100-250 times), even more preferably 250-1000 times, and most preferably at least 1000 times the LD50 value with respect to cancer cells at 2.5×10^3 and a reaction time of 48 hours.

(5) Furthermore, the antibody or functional fragment thereof according to the present invention is capable of inhibiting the growth of tumors (for example, those originating in Colo205 cells transplanted into nude mice), or causing regression of tumors. In this case, the period of time over which the growth of tumor cells can be suppressed or the period of time over which tumors can be caused to regress when the antibody or functional fragment thereof according to the present invention is administered is at least 9 days, preferably at least 11 days, more preferably at least 13 days, subsequently preferable in the order of at least 30 days and at least 60 days, and most preferably at least 120 days. Additionally, the dosage of the antibody or functional fragment thereof according to the present invention to be administered to test animals (for example, cancerous lab animals with a body weight of 20 g) carrying tumors is about 0.1 μg/body (5 μg/kg) to 100 μg/body (5 mg/kg). For example, the dosage can be 100 μg/body or 5 mg/kg, preferably 20 μg/body or 1 mg/kg, more preferably 4 μg/body or 200 μg/kg, and even more preferably 1 μg/body or 50 μg/kg. Additionally, it is possible to administer 0.5 μg/body (25 μg/kg). The administration frequency can be a frequency of 1 to 3

times per week or every other day.

Furthermore, the anti-tumor effects of the antibody (for example, 0304 antibody or E-11-13 antibody) or functional fragment thereof according to the present invention are as follows.

(a) Capable of causing shrinkage of a tumor of on average at least 14% four days after an initial treatment, when administered at a concentration of 20 μg/mouse to 4-6 week old cancerous mice having tumors of 100 mm^3. In this case, the shrinkage of the tumor of on average at least 14% is sustained for at least 7 days.

(b) Capable of causing shrinkage of a tumor of on average at least 65% four days after an initial treatment, when administered at a concentration of 20 μg/mouse to 4-6 week old cancerous mice having tumors of 100 mm^3.

(c) Capable of causing shrinkage of a tumor of on average at least 80% seven days after an initial treatment, when administered at a concentration of 20 μg/mouse to 4-6 week old cancerous mice having tumors of 100 mm^3. In this case, the shrinkage of the tumor of on average at least 80% is sustained for at least 4 days.

(d) Capable of causing shrinkage of a tumor of on average at least 45% three days after an initial treatment, when administered at a concentration of 25 μg/mouse to 12 week old cancerous mice having tumors of 100 mm^3.

(e) Capable of causing shrinkage of a tumor of on average at least 65% five days after an initial treatment, when administered at a concentration of 25 μg/mouse to 12 week old cancerous mice having tumors of 100 mm^3. In this case, the shrinkage of the tumor of on average at least 65% is sustained for at least 27 days.

(f) Capable of causing shrinkage of a tumor of on average at least 39% four days after an initial treatment, when administered at a concentration of 20 μg/mouse to 4-6 week old cancerous mice having tumors of 300 mm^3. In this case, the shrinkage of the tumor of on average at least 39% is sustained for at least 14 days.

Examples of tumors include at least one type chosen from among colon cancer, colorectal cancer, lung cancer, mammary cancer, brain tumors, melanoma, renal cell cancer, cystic cancer, leukemia, lymphoma, T-cell lymphoma, multiple myeloma, gastric cancer, pancreatic cancer, cervical cancer, endometrial cancer, ovarian cancer, esophagal cancer, hepatic cancer, squamous cancer, skin cancer, urethral cancer, prostate cancer, chorionic cancer,

pharyngeal cancer, laryngeal cancer, pleural cancer, androblastoma, endometrial hyperplasia, endometriosis, embryoma, fibrosarcoma, Kaposi sarcoma, hemangioma, cavernous hemangioma, angioblastoma, retinoblastoma, astrocytoma, neurofibroma, oligodendroglioma, medulloblastoma, neuroblastoma, neuroglioma, rhabdomyosarcoma, glioblastoma, osteogenic sarcoma, leiomyosarcoma, goiter and Wilms tumors.

(6) An antibody or functional fragment thereof, having an amino acid sequence at a mature portion comprising the heavy chain variable domain and light chain variable domain of an antibody produced by hybridoma E-11-13 as respectively indicated by Sequences Nos. 17 and 19, the heavy chain variable domain and light chain variable domain of an antibody produced by hybridoma L-30-10 as respectively indicated by Sequences Nos. 21 and 23, the heavy chain variable domain and light chain variable domain of an antibody produced by hybridoma H-48-2 as respectively indicated by Sequences Nos. 25 and 27, the heavy chain variable domain and light chain variable domain of an antibody produced by hybridoma 0304 as respectively indicated by Sequences Nos. 29 and 31, and the heavy chain variable domain and light chain variable domain of an antibody produced by hybridoma KMTR1 as respectively indicated by Sequences Nos. 33 and 35.

The above-described antibody or functional fragment thereof can have an amino acid sequence at a mature portion comprising the heavy chain variable domain and light chain variable domain coded by a nucleic acid sequence isolated from hybridoma E-11-13 as respectively indicated by Sequences Nos. 16 and 18, the heavy chain variable domain and light chain variable domain coded by a nucleic acid sequence isolated from hybridoma L-30-10 as respectively indicated by Sequences Nos. 20 and 22, the heavy chain variable domain and light chain variable domain coded by a nucleic acid sequence isolated from hybridoma H-48-2 as respectively indicated by Sequences Nos. 24 and 26, the heavy chain variable domain and light chain variable domain coded by a nucleic acid sequence isolated from hybridoma 0304 as respectively indicated by Sequences Nos. 28 and 30, and the heavy chain variable domain and light chain variable domain coded by a nucleic acid sequence isolated from hybridoma KMTR1 as respectively indicated by Sequences Nos. 32 and 34.

(7) A hybridoma producing a monoclonal antibody binding to TRAIL-R2, chosen from the group consisting of E-11-13, H-48-2, L-30-10, N-18-12, W-40-5, X-14-4, X-51-12, F-4-8, G-3-10, 0304 and KMTR1.

(8) A method for producing an anti-TRAIL-R2 monoclonal antibody, characterized by cultivating the above-described hybridoma; and collecting antibodies binding to TRAIl-R2 from the resulting culture.

(9) A method for producing an anti-TRAIL-R2 monoclonal antibody, characterized by isolating a gene coding for an anti-TRAIL-R2 monoclonal antibody from the above-described hybridoma; constructing an expression vector having said gene; introducing said expression vector into a host and causing expression of said monoclonal antibody; and collecting the anti-TRAIL-R2 monoclonal antibody from the resulting host, conditioned medium of the host or secretions from the host.

The host can be chosen from the group consisting of *E. coli*, yeast cells, insect cells, mammalian cells and vegetable cells as well as mammals.

(10) A preventive or therapeutic agent for tumors, containing the above-described antibody or functional fragment thereof as an active ingredient.

Here, the tumor can be of at least one type chosen from among colon cancer, colorectal cancer, lung cancer, mammary cancer, brain tumors, melanoma, renal cell cancer, cystic cancer, leukemia, lymphoma, T-cell lymphoma, multiple myeloma, gastric cancer, pancreatic cancer, cervical cancer, endometrial cancer, ovarian cancer, esophagal cancer, hepatic cancer, squamous cancer, skin cancer, urethral cancer, prostate cancer, chorionic cancer, pharyngeal cancer, laryngeal cancer, pleural cancer, androblastoma, endometrial hyperplasia, endometriosis, embryoma, fibrosarcoma, Kaposi sarcoma, hemangioma, cavernous hemangioma, angioblastoma, retinoblastoma, astrocytoma, neurofibroma, oligodendroglioma, medulloblastoma, neuroblastoma, neuroglioma, rhabdomyosarcoma, glioblastoma, osteogenic sarcoma, leiomyosarcoma, goiter and Wilms tumors.

Herebelow, the present invention shall be described in detail. The specification and/or drawings of Japanese Patent Application No. 2001-150213 (filed May 18, 2001), Japanese Patent Application No. 2001-243040 (filed August 9, 2001) and Japanese Patent Application No. 2001-314489 (filed October 11, 2001), whose priority is claimed by the present application, are incorporated into the present specification.

There have been reports that anti-TRAIL-R1 and R2 monoclonal antibodies have activity for inducing apoptosis in cancer cells [see Griffith, T. S. *et al.* (1999) *J. Immunol.* 162,

2597-2605; Chuntharapai, A. *et al.* (2001) *J. Immunol.* 166, 4891-4898]. However, these antibodies were of murine origin.

Additionally, there is concern as to the risk of damage to healthy human hepatocytes, which is a problem associated with recombinant human TRAIL proteins.

Surprisingly, however, the novel human anti-TRAIL-R2 monoclonal antibodies of the present invention were discovered not to have any side effects leading to damage, not only with respect to cells from healthy human tissue, but also with respect to healthy hepatocytes which were held to be at risk of cell damage due to recombinant human TRAIL proteins. By obtaining these novel anti-TRAIL-R2 monoclonal antibodies, the present inventors succeeded for the first time in the world to produce antibodies having the advantages of both safety and the possibility of improved therapeutic effect. These monoclonal antibodies are preferably human antibodies, which obviates concerns as to antigenicity as can be a problem with murine antibodies.

While immunoglobulins G (IgG), A (IgA), E (IgE) and M (IgM) are all suitable forms for the antibody, IgG is usually preferred.

Herebelow, the present invention shall be described in detail by clarifying the meanings of the terms used in the present invention.

1. TRAIL and Antibodies Thereof

The antibodies of the present invention are antibodies against the receptors (TRAIL-R) of tumor necrosis factor (TNF)-related apoptosis-inducing ligands (TRAIL), of which there are (i) antibodies reactive with TRAIL-R1, (ii) antibodies reactive with TRAIL-R2, and (iii) antibodies reactive with both TRAIL-R1 and TRAIL-R2. In the present invention, the antibodies of (i) shall often be referred to as "anti-TRAIL-R1 antibodies", and the antibodies of (ii) and (III) shall often be referred to as "anti-TRAIL-R2 antibodies". Additionally, when referring collectively to both TRAIL receptors TRAIL-R1 and TRAIL-R2 in the present specification, they shall often be called "TRAIL-R1 and R2". Therefore, when describing, for example, "TRAIL-R1 and R2 expression vectors" (see Examples below), this shall refer to both TRAIL-R1 expression vectors and TRAIL-R2 expression vectors.

In the present invention, "antibodies" shall refer to antibodies or portions of antibodies, including function fragments of these antibodies, reactive with human TRAIL-R1 and R2 as defined above or portions thereof. "Functional fragments" refer to portions of

antibodies (partial fragments) having at least one type of action against the antigens of the antibodies, specific examples including $F(ab')_2$, Fab', Fab, Fv, disulphide-bonded Fv, single-chain Fv (scFv) and polymers thereof (D. J. King, *Applications and Engineering of Monoclonal Antibodies*, 1998, T. J. International Ltd.).

For the purposes of the present invention, "human antibodies" shall refer to antibodies which are the expression products of human antibody genes.

Antibodies of the present invention can include, for example, various antibodies having the property of inducing apoptosis in cancer cells expressing human TRAIL-R1 and R2, such as are described in Example 7 below.

The antibodies of the present invention include monoclonal antibodies composed of heavy and/or light chains wherein the amino acids sequences of the heavy and/or light chains forming the antibodies are such that one or more amino acids have been deleted, substituted or added to the amino acid sequences. Methods for introducing partial modifications (deletions, substitutions, insertions, additions) to the amino acids as described above can include methods of partially modifying the base sequences coding for the amino acid sequences. Such a partial modification of a base sequence can be performed by means of conventionally known processes such as site-specific mutagenesis (*Proc. Natl. Acad. Sci. USA*, 1984, vol. 81:5662). Here, the antibodies are immunoglobulins wherein all domains forming the immunoglobulins, including the heavy chain variable region and heavy chain constant region as well as the light chain variable region and the light chain constant region, are from genes coding for the immunoglobulins.

The antibodies of the present invention include antibodies having of all immunoglobulin classes and isotypes.

The anti-TRAIL-R1 and R2 antibodies of the present invention can be produced by the following production methods. For example, non-human mammals such as human antibody-producing transgenic mice or the like are immunized with a conjugate of human TRAIL-R1 and R2 as defined above or a portion thereof with an appropriate substance for increasing the antigenicity of antigens (*e.g.* bovine serum albumin), along with an immunostimulatory agent (such as complete or incomplete Freund's adjuvant). Alternatively, they can be immunosensitized by incorporating a gene coding for human TRAIL-R1 or a gene coding for human TRAIL-R2 so as to result in animal cells excessively expressing TRAIL-R1 and TRAIL-R2 at the cell surface. The monoclonal antibodies can be obtained by cultivating the

hybridomas obtained by fusing antibody-producing cells obtained from immunized animals with myeloma cells not having the ability to produce their own antibodies, and selecting the clones producing monoclonal antibodies exhibiting a specific affinity to the antigens used for immunization.

The antibodies of the present invention include those which have been converted to a different subclass by genetic engineering modifications which are conventionally known to those skilled in the art. For example, the subclasses of the antibodies of the present invention can be converted to IgG2 or IgG4 to acquire antibodies with low rates of binding with Fc receptors. Conversely, the subclasses of the antibodies of the present invention can be converted to IgG1 or IgG3 to acquire antibodies with high rates of binding with Fc receptors. Furthermore, it is also possible to change the rates of binding to Fc receptors by artificially modifying the amino acid sequences at the constant regions of the antibodies or binding with constant region sequences having such sequences. Additionally, the antibodies of the present invention can be bound to radionuclides of iodine, yttrium, indium and technetium (J. W. Goding, *Monoclonal Antibodies: principles and practice*, 1993, Academic Press), bacterial toxins such as pyocyanin, diphtheria toxin and ricin, chemotherapy agents such as methotrexate, mitomycin and calicheamycin (D. J. King, *Applications and Engineering of Monoclonal Antibodies*, 1998, T. J. International Ltd.; M. L. Grossbard, *Monoclonal Antibody-based Therapy of Cancer*, 1998, Marcel Dekker Inc.), as well as prodrugs such as Maytansinoid (Chari *et al.*, *Cancer Res.*, 1992, vol. 52:127; Liu *et al.*, *Proc. Natl. Acad. Sci. USA*, 1996, vol. 93:8681), in order to further enhance the effectiveness in treating diseases such as cancer.

Additionally, the present inventors discovered that among the antibodies of the present invention having the property of binding to TRAIL-R2 but not binding to TRAIL-R1, there are those which do not cause damage to human hepatocytes. Accordingly, the present invention offers a method of producing anti-TRAIL-R2 antibodies which do not damage hepatocytes, characterized by comprising a step of selecting, from a group of antibodies binding to trail-R2, those which do not bind to TRAIL-R1. However, the antibodies of the present invention which do not cause damage to hepatocytes are not limited to those having the property of binding to TRAIL-R2 but not binding to TRAIL-R1.

In the present invention, the production of the monoclonal antibodies involves the following processing steps. That is, (1) purification of biopolymers and/or production of cells excessively expressing antigen proteins on the cell surface (these biopolymers and/or cells are

used as immunogens); (2) after immunizing animals by injection of antigens, collecting blood to assay the antibody titer and determining the time of splenectomy or the like to prepare antibody-producing cells; (3) preparing myeloma cells; (4) fusion of antibody-producing cells and myeloma cells, (5) selection of hybridoma groups producing the objective antibodies; (6) division to unicellular clones (cloning); (7) depending on the situation, cultivating the hybridomas to produce large quantities of the monoclonal antibodies, or breeding animals in which the hybridomas have been transplanted; and (8) studying the bioactivity and recognition specificity of the thusly produced monoclonal antibodies, or assaying the properties as a labeling reagent.

Herebelow, the method of producing the anti-TRAIL-R1 and R2 monoclonal antibodies shall be described in detail by following the above-described steps, but the methods for producing the antibodies are not limited to these, and it is also possible, for example to use antibody-producing cells aside from splenocytes along with the myeloma cells.

(1) Purification of Antigens

As the antigens, it is possible to use fusion proteins of the extracellular domain of human TRAIL-R1 and R2 and the Fc domain of human IgG (hereinafter referred to as TRAIL-R1-hFc and TRAIL-R2-hFc). TRAIL-R1-hFc and TRAIL-R2-hFc can be obtained by integrating DNA encoding the fusion protein of TRAIL-R1 or R2 and the Fc domain of human IgG into an expression vector of an animal cell, introducing the expression vector into an animal cell, and purifying from the conditioned medium of the resulting transformed cell line. Alternatively, it is possible to use TRAIL-R1-hFc and TRAIL-R2-hFc as commercially available from ALEXIS. Additionally, TRAIL-R1 and R2 present in the cell membranes of human cell lines can be used as the antigens by purifying them. Furthermore, since the primary structures of TRAIL-R1 and R2 are publicly known [see Pan, G. *et al.* (1997) *Science* 276, 111-113 and *Science* 277, 815-818; and Walczak, H. *et al.* (1997) *EMBO J* 16 (17) 5386-5397], peptides can be chemically synthesized from the amino acid sequences of TRAIL-R1 and R2, and these used as antigens.

Additionally, effective immunogens can be obtained by introducing expression vectors pEF-TRAIL-R1delta and pEF-TRAIL-R2delta, containing TRAIL-R1 and R2 with the death domain in the intracellular region and the amino acids on the C-terminus side of the death domain removed from the full-length human TRAIL-R1 and R2 (hereinafter referred to as "TRAIL-R1 and R2delta"), into an L929 cell to result in a cell that excessively expressed

TRAIL-R1 and R2delta at the cell surface. pEF-TRAIL-R1delta and pEF-TRAIL-R2delta can be produced by integrating respectively DNA encoding the human TRAIL-R1delta protein and DNA encoding the human TRAIL-R2delta protein into an expression vector pEFneo for animal cells [see Ohashi, H. *et al.* (1994) *Proc. Natl. Acad. Sci.* 91, 158-162]. However, the DNA encoding TRAIL-R1 and R2, the vector, the host and the like are not necessarily restricted to the above.

Specifically, L929 cells excessively expressing human TRAIL-R1 and R2 delta at the cell surface can be produced by cultivating a transformed cell line obtained by transforming L929 cells with pEF-TRAIL-R1 and R2 delta, then identifying the trait of neomycin resistance obtained in cells into which the pEFneo vector has been inserted, and confirming the expression of TRAIL-R1 and R2 delta using caprine anti-TRAIL-R1 and R2 polyclonal antibodies (DAKO).

(2) Antibody-Producing Cell Preparation Step

Laboratory animals are immunized with an immunogen formed by mixing the antigen obtained in (1) with an adjunct such as a complete or incomplete Freund's adjuvant or potassium alum. As the laboratory animals, it is most preferable to use transgenic mice having the ability to produce human antibodies. Such mice are described in a publication by Tomizuka *et al.* [Tomizuka *et al., Proc Natl Acad Sci USA*, 2000, vol. 97:722].

The method of administering the immunogen for immunization of the mice can be any of hypodermic injection, intraperitoneal injection, intravenous injection, intradermal injection, intramuscular injection and footpad injection, of which hypodermic injection, intraperitoneal injection, footpad injection and intravenous injection are preferable.

Immunization can be performed just once, or repeated a plurality of times at appropriate intervals (preferably at intervals of from 3 days to 1 or 2 weeks). The effect of the succeeding operations can be enhanced by thereafter measuring the antibody titer against the antigen in the serum of the immunized animal, and using the animals with a sufficiently high antibody titer as the supply source of the antibody-producing cells. Generally, it is preferable to use the antibody-producing cells from animals 3-5 days after their final immunization for the cell fusion to follow.

Examples of the method for measuring the antibody titer used here include various publicly known techniques such as a radioimmunoassay (hereinafter referred to as RIA), enzyme-linked immunosorbent assay (hereinafter referred to as ELISA), fluorescent antibody assay and passive hemagglutination assay, of which RIA and ELISA are preferable in

consideration of the assay sensitivity, speed, accuracy and possibility of automation of operations.

By using, for example, the ELISA method, the antibody titer measurement for the present invention can be performed by the procedure described below. First, purified or partially purified recombinant human TRAIL-R1 and R2 are bound to the surface of the solid phase in a 96-hole ELISA plate or the like, and the parts of the solid phase to which the antigens are not bound are blocked by a protein such as bovine serum albumin (hereinafter referred to as "BSA") unrelated to the antigen. After rinsing the surface, it is brought into contact with a serially diluted sample (such as murine serum) as a primary antibody and the anti-TRAIL-R1 and R2 antibodies in the sample are bound to the above-described antigen. Further, an antibody to the human antibody which is enzyme-labeled is added as a secondary antibody which is bound to the human antibody, and after rinsing, the substrate of the enzyme is added. The changes in the light absorption due to discoloration based on decomposition of the substrate is measured to calculate the antibody titer.

(3) Myeloma Preparation Step

As the myeloma, it is possible to use cells which are not capable of producing their own antibodies, from mammals such as mice, rats, guinea pigs, hamsters, rabbits or humans, of which most preferable for general use are established cell lines from mice, such as 8-azaguanine resistant mouse (derived from BALB/c) myeloma strain lines P3X63Ag8U. 1 (P3-U1) [Yelton, D. E. *et al., Current Topics in Microbiology and Immunology*, 81, 1-7 (1978)], Pc/NSI/1-Ag4-1 (NS-1) [Kohler, G. *et al., European J. Immunology*, 6, 511-519 (1976)], Sp2/0-Ag14 (SP-2) [Shulman, M. *et al., Nature*, 276, 269-270 (1978)], P3X63Ag8. 653 (653) [Kearney, J. F. *et al., J. Immunology*, 123, 1548-1550 (1979)], P3X63Ag8 (X63) [Horibata, K. and Harris, A. W., *Nature*, 256, 495-497 (1975)]. These cell lines are passage cultured using an appropriate culture medium such as an 8-azaguanine medium [a culture medium formed by adding glutamine, 2-mercaptoethanol, gentamicin and fetal calf serum (hereinafter referred to as "FCS") to an RPMI-1640 culture medium and further adding 8-azaguanine], an Iscove's Modified Dulbecco's Medium (hereinafter referred to as "IMDM"), or a Dulbecco's Modified Eagle Medium (hereinafter referred to as "DMEM"), but are passage cultured in a normal culture medium (*e.g.* a DMEM culture medium containing 10% FCS) 3-4 days prior to cell fusion, to obtain at least 2×10^7 cells by the day of fusion.

(4) Cell Fusion

The antibody-producing cell is a plasmocyte and a lymphocyte which is a precursor cell thereof, which can be taken from any part of the body, generally from the spleen, lymph nodes, bone marrow, tonsils, peripheral blood or an appropriate combination thereof, of which splenocytes are most generally used.

After the final immunization, the portions of the mice which exhibit a predetermined antibody titer containing the antibody-producing cells, such as the spleen, are extirpated, to prepare splenocytes which are antibody-producing cells. The method which is currently most commonly used to fuse these splenocytes with the myelomas obtained in step (3) is the method of using polyethylene glycol which has relatively low cytotoxicity and has simple fusion operations. This method can, for example, consist of the following procedure.

The splenocytes and myelomas are well-rinsed with a serum-free medium (such as DMEM), or phosphate buffer saline solution (hereinafter referred to as "PBS"), mixed so that the ratio of splenocytes to myelomas cells is about 5 : 1 to 10 : 1, then centrifuged. After discarding the supernatant and loosening the precipitated cell group, it is instilled while stirring onto a serum-free culture medium containing 1 ml of 50% (w/v) polyethylene glycol (molecular weight 1000-4000). Then, 10 ml of serum-free culture medium are slowly added, and the result centrifuged. The supernatant is again discarded, and the precipitated cells are suspended in a normal culture medium containing suitable amounts of hypoxanthine-aminopterin-thymidine (hereinafter referred to as "HAT") solution and human interleukin-6 (hereinafter referred to as "IL-6"), which is distributed in the wells of a culture plate (hereinafter referred to as "plate"), and cultivated for about 2 weeks in the presence of 5% carbon dioxide gas at 37 °C. In the meantime, HAT medium is supplemented as needed.

(5) Selection of Hybridoma Group

If the above-described myeloma cells are a 8-azaguanine resistant line, in other words, if they deficient in hypoxanthine-guanine-phosphoribosyl transferase (HGPRT), then the myeloma cells which did not fuse and the fusion cells between myeloma cells will not be able to survive in a HAT-containing culture medium. On the other hand, the fusion cells between antibody-producing cells and hybridomas between the antibody-producing cells and myeloma cells will be able to survive, but the lifetimes of the fusion cells between antibody-producing

cells will be limited. Therefore, by continuing to cultivate them in a HAT-containing culture medium, only the hybridomas between the antibody-producing cells and the myeloma cells will live on, thus ultimately selecting out the hybridomas.

The hybridomas which are grown in the form of a colony are transferred from the HAT medium to a culture medium without aminopterin (hereinafter referred to as "HT medium"). Thereafter, some of the supernatant of the medium is collected, and measured for the anti-TRAIL-R1 and R2 antibody titer by means of the ELISA method or the like. However, if the above-described fusion proteins are used as the antigens for the ELISA assay, then an operation is required to remove clones producing antibodies which specifically bind to the Fc domain of human IgG so that they will not be selected. It is possible to check for the presence of such clones, for example, by means of an ELISA assay using the Fc domain of human IgG as the antigen.

While we have given examples of methods using an 8-azaguanine resistant cell line above, it is also possible to use other cell lines depending on the method of selection of hybridomas, in which case the compositions of the culture media used will also change.

(6) Cloning Step

Hybridomas found to produce specific antibodies by measuring the antibody titer with the same method as described in (2) are transferred to a separate plate and cloned. Examples of cloning methods include a limiting dilution method wherein the hybridomas are diluted to one per well of the plate for cultivation, a soft agar method where they are cultivated in a soft agar culture medium and the colonies are recovered, a method in which the cells are extracted one at a time by means of a micromanipulator and cultivated, and a "soft clone" method in which a single cell is separated by means of a cell sorter, of which the limiting dilution method is convenient and therefore often used.

The wells which are found to have acceptable antibody titers are cloned 2-4 times, for example, by means of the limiting dilution method, and those for which a stable antibody titer have been acknowledged are selected as anti-TRAIL-R1 and R2 monoclonal antibody-producing hybridoma lines.

The mouse-mouse hybridoma H-48-2 which is the human anti-TRAIL-R2 monoclonal antibody-producing cell of the present invention was internationally deposited at the International Patent Organism Depositary at the National Institute of Advanced Industrial

Science and Technology (AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan) dated May 18, 2001. The international deposit number is FERM BP-7599. Additionally, the hybridoma E-11-13 was internationally deposited under the deposit number FERM BP-7698, hybridoma F-4-8 under the deposit number FERM BP-7699 and hybridoma L-30-10 under the deposit number FERM BP-7700, dated August 8, 2001. Additionally, hybridoma 0304 was internationally deposited under the deposit number FERM BP-8037 and hybridoma KMTR1 under the deposit number FERM BP-8038, dated May 10, 2002. Accordingly, in order, for example, to prepare antibodies using a mouse-mouse hybridoma, the steps up to step (6) can all be omitted and the preparation of the antibody begun from the following step (7). Additionally, they can be cultivated *in vivo* in the ascites of mice, then isolated from the ascites.

(7) Preparation of Monoclonal Antibodies by Hybridoma Cultivation

The hybridomas which have been cloned are transferred from the HT culture medium to a normal culture medium and cultivated. Mass cultures can be made using a rotating culture using a large culture bottle or by a spinner culture. By purifying the supernatant of this mass culture by means of methods commonly known to those skilled in the art such as gel filtration, it is possible to obtain anti-TRAIL-R1 and R2 monoclonal antibodies containing the preventive or therapeutic agent of the present invention as the active ingredient. Additionally, by growing the hybridomas in the peritoneal cavities of the same line of mice (such as BALB/c) or Nu/Nu mice, rats, guinea pigs, hamsters or rabbits, it is possible to obtain ascites containing large quantities of anti-TRAIL-R1 and R2 monoclonal antibodies containing the preventive or therapeutic agent of the present invention as the active ingredient. A commercially available monoclonal antibody purifying kit (e.g. MabTrap GII kit from AMERSHAM PHARMACIA BIOTECH) or the like can be used as a convenient method of purification.

The monoclonal antibody obtained in this way has a high antigen specificity to human TRAIL-R1 and R2.

(8) Monoclonal Antibody Assay

Assays of isotypes and subclasses of monoclonal antibodies obtained in this way can be performed as follows. First, the Ouchterlony method, the ELISA method and RIA method can be given as examples of assay methods. Although the Ouchterlony method is convenient, it requires manipulation of the concentration if the concentration of the monoclonal antibody is

low.

On the other hand, when using the ELISA method or the RIA method, the conditioned medium is reacted directly with an antigen-bound solid phase, then antibodies corresponding to the various immunoglobulin isotypes and subclasses are used as secondary antibodies to enable identification of isotypes and subclasses of the monoclonal antibodies.

Furthermore, the quantitation of proteins can be performed by the Folin-Lowry method or a method of calculation from the absorption at 280 nm [1.4 (OD280) = immunoglobulin 1 mg/ml].

The identification of monoclonal antibody-recognizing epitopes is performed in the following manner. First, various partial structures of molecules recognizing monoclonal antibodies are prepared. As methods for preparing the partial structures, there are methods of using publicly known oligopeptide synthesis technology to make various partial peptides of these molecules, and methods of integrating a DNA sequence coding for a desired partial peptide into a suitable expression plasmid using genetic recombination technology to produce it inside or outside a host such as *E. coli* or the like, but it is common to use a combination of both methods for the above-given purposes. For example, a group of polypeptides which have been made consecutively shorter by an appropriate length taken from the C-terminus or N-terminus of an antigen protein are produced using genetic recombination technology which is conventionally known to those skilled in the art, then their reactivity to monoclonal antibodies is analyzed to result in an approximation of the recognizing portions.

Thereafter, the oligopeptides of the corresponding portions or variants thereof are further synthesized using oligopeptide synthesis technology conventionally known to those skilled in the art, and the monoclonal antibodies contained as the active ingredients of the preventive or therapeutic agent of the present invention are tested for their binding ability to these peptides, or the competitive inhibition activity of the peptides with respect to binding between these monoclonal antibodies and antigens is studies to restrict the epitopes. As a convenient method of obtaining various oligopeptides, it is possible to use various commercially available kits (*e.g.* SPOTs kit (Genosys Biotechnologies), Multipin peptide synthesis kit (Chiron) using the multipin synthesis method, *etc.*).

Additionally, genes coding for human monoclonal antibodies can be cloned from antibody-producing cells such as hybridomas, and integrated with appropriate vectors, then incorporated into hosts (*e.g.* mammalian cell lines, *E. coli*, yeasts, insect cells, vegetable cells or

the like), thus to prepare recombinant antibodies produced using gene recombination technology (P. J. Delves, *Antibody Production Essential Techniques*, 1997, Wiley; P. Shepherd and C. Dean, *Monoclonal Antibodies*, 2000, Oxford University Press; J. W. Goding, *Monoclonal Antibodies: Principles and Practice*, 1993, Academic Press).

In order to prepare genes coding for monoclonal antibodies from hybridomas, a method of preparing DNA coding respectively for the light chain variable domain, light chain constant domain, heavy chain variable domain and heavy chain constant domain using the PCR method or the like can be employed. An anti-TRAIL-R antibody gene or oligoDNA designed from the amino acid sequence can be used as the primer, and DNA prepared from a hybridoma can be used as the template. These DNA are integrated into a single appropriate vector, which is then incorporated into a host for expression, or these DNA can be integrated into separate appropriate vectors and co-expressed.

The vectors may be phages or plasmids capable of self-replication in a host microbe. Examples of such plasmid DNA include plasmids from *E. coli*, *B. subtilis* or yeasts, and examples of phage DNA include λ phage.

The host used for the transformation is not particularly restricted as long as it is capable of expressing the desired gene. Examples include bacteria (*E. coli*, *B. subtilis etc.*), yeasts, animal cells (COS cells, CHO cells *etc.*) and insect cells.

Methods for incorporating genes into hosts are publicly known, and any method can be used (*e.g.* methods using calcium ions, electroporation, spheroplasts, lithium acetate, cocium phosphate, lipofection *etc.*). Additionally, methods for incorporating genes into animals as described below include microinjection, methods for incorporating genes into ES cells using electroporation or lipofection, and nuclear transplantation.

In the present invention, anti-TRAIL-R antibodies can be obtained by cultivating transformants and collecting them from the culture. The term "culture" refers to any one of (a) a conditioned medium, (b) culture cells, culture microbes in whole or crushed form, or (c) secretions of the transformants. In order to cultivate the transformants, a static culture method or a roller bottle culture method is employed using a medium appropriate to the host used.

If the desired proteins are produced inside the microbes or cells, the antibodies are obtained by crushing the microbes or cells after cultivation. If the desired antibodies are produced outside the microbes or cells, the culture fluid is directly used or after removing the microbes or cells by means of centrifugation or the like. Subsequently, common biochemical

methods using various types of chromatography used for isolating and purifying proteins are used alone or in combination to isolate and purify the desired antibodies from the culture.

Furthermore, transgenic animal production technology can be used to produce animal hosts carrying the gene for the desired antibody incorporated within the endogenous genes, such as transgenic cattle, transgenic goats, transgenic sheep or transgenic pigs, and monoclonal antibodies from the antibody genes can be acquired in large quantities from the milk secreted by such transgenic animals (Wright, G. *et al.* (1991) *Bio/Technology* 9, 830-834). In order to cultivate hybridomas *in vitro*, hybridomas can be grown, maintained and preserved in accordance with various conditions such as the purpose of the research and method of cultivation using known nutrient media such as are used for producing monoclonal antibodies in the conditioned medium, or using various nutrient media derived from base media that are already known.

(9) Properties of Antibodies

The antibodies of the present invention have the following functional properties a)-c), and each of the properties can be confirmed, for example, using the method given in each paragraph.

a) They have activity such that when human cancer cells are cultivated, and the survival rate of the cells is determined after the antibodies of the present invention are added to the culture, apoptosis has been induced in the cancer cells expressing TRAIL-R1 and/or R2.

b) When cells from healthy human tissue are cultivated, and the survival rate of the cells is determined after the antibodies of the present invention are added to the culture, the healthy cells which express TRAI+-R1 and/or R2 are unaffected.

c) When human hepatocytes are cultivated, and the survival rate of the cells is determined after the antibodies of the present invention are added to the culture, the hepatocytes are not damaged.

The apoptosis-inducing activity of the antibodies of the present invention can be represented using the LD50 value (the concentration of antibodies causing death in half the cells under predetermined experimental conditions) as an index. The LD50 value should be 100 μg/ml or less, preferably 10μg/ml or less, more preferably 0.7μg/ml or less, even more preferably 0.02-0.11μg/ml, and most preferably 0.02μg/ml or less under the experimental conditions to be described.

Additionally, expressions such as "not affecting healthy cells" and "not causing damage to hepatocytes" refer to the fact that the antibodies of the present invention do not have very high apoptosis-inducing activity with respect to healthy cells (human hepatocytes). When taking the LD50 value as the index, it should be at least 0.01μg/ml, preferably at least 0.1μg/ml, more preferably 2-10μg/ml, even more preferably 10-24μg/ml, and most preferably at least 24μg/ml under the experimental conditions to be described.

While the antibodies of the present invention have the activity of any one of the above-given paragraphs a)-c), they should preferably be substances having the novel property of having both the activity of paragraph a), *i.e.* activity to induce apoptosis in cancer cells, and the activity of paragraphs b) and c), *i.e.* activity of not causing damage to healthy cells, particularly healthy hepatocytes. Therefore, the antibodies of the present invention are useful as ingredients to be contained in preventive and therapeutic agents for malignant tumors.

The apoptosis-inducing activity with respect to healthy cells or cancer cells can be represented by using the LD50 value as an index. The LD50 value in the present invention can be computed as follows. Healthy cells (*e.g.* human hepatocytes) or cancer cells (*e.g.* human colon cancer cell line Colo205; ATCC CCL-222) are cultivated, the antibodies of the present invention are added to the culture, and the cell survival rate after the passage of a certain period of time is measured by an MTT assay (Green, L. M. *et al., J. Immunological Methods*, 70:257-268 (1984)) or an LDH assay.

The antibody concentration corresponding to a survival rate of 50% as determined from a graph plotting the survival rate against the concentration of antibody added is taken as the LD50 value.

The LD50 value can either be read from the graph or determined from the equation of the plotted curve using recursive computation.

In a test for Colo205 cancer cells, a 96-hole flat-bottom plate with 100 μl of culture medium per hole was inoculated with 2.5×10^3 cells which were cultivated at 37 °C in the presence of 5% CO_2. The following day, an antibody was added, and after letting stand for 48 hours in the above-stated environment, the cell survival rate was measured (total amount of reactant: 110-120 μl). For the purposes of the present invention, the above-described conditions shall be referred to as "cell number 2.5×10^3 and reaction time 48 hours".

In a test for healthy cells (hepatocytes), a 96-hole flat-bottom plate with 100 μl of culture medium per hole was inoculated with 7.5×10^4 cells which were cultivated at 37 °C in

the presence of 5% CO_2. The following day, an antibody was added, and after letting stand for 48 hours in the above-stated environment, the cell survival rate was measured (total amount of reactant: 110-120 μl). For the purposes of the present invention, the above-described conditions shall be referred to as "cell number 7.5×10^4 and reaction time 48 hours".

The antibodies of the present invention include those having a property such that the LD50 value with respect to healthy cells (human hepatocytes), when measuring the LD50 value under the above-given conditions, is for example, at least 0.01μg/ml (10 ng/ml), and preferably at least 0.1μg/ml. Since the level of safety can be considered to be greater as the LD50 value with respect to healthy cells becomes greater, antibodies having an LD50 value of 2-100μg/ml are even more preferable. Additionally, the antibodies of the present invention include those having a property such that the LD50 value with respect to cancer cells, when measuring the LD50 value under the above-given conditions, is for example, 100 μg/ml or less, and preferably 0.7μg/ml or less. Since the tumoricidal activity can be considered to be stronger as the LD50 value with respect to cancer cells becomes smaller, antibodies having an LD50 value of 0.02-0.11 μg/ml are even more preferable. IN particular, the E-11-13 antibody, L-30-10 antibody and KMTR1 antibody of the present invention all have properties such that the LD50 value with respect to human hepatocytes is at least 2-100μg/ml (e.g. 2-24μg/ml, preferably 100μg/ml), and the LD50 value with respect to cancer cells is 0.02-0.11μg/ml, thus exhibiting both safety toward healthy cells and an apoptosis-inducing effect toward tumor cells. More surprisingly, the antibodies of the present invention were able to considerably inhibit the proliferation of cancer cells in a cancerous mouse model.

Here, a comparison shall be made between the LD50 value with respect to healthy cells when measured under the condition "cell number 7.5×10^4 and reaction time 48 hours" and the LD50 value with respect to cancer cells when measured under the condition "cell number 2.5×10^3 and reaction time 48 hours". As described above, the level of safety becomes higher as the LD50 value with respect to healthy cells becomes higher, and the tumoricidal activity becomes stronger as the LD50 value with respect to cancer cells becomes lower, so that when comparing the LD50 value with respect to healthy cells and the LD50 value with respect to cancer cells, a high value for their ratio can be considered to be more useful (the safety is greater and the apoptosis-inducing activity with respect to cancer cells is stronger). When taking the ratio of LD50 values of healthy cells to cancer cells as N/C (how many times greater is the LD 50 value with respect to healthy cells as compared to the LD50 value with respect to

cancer cells), the antibodies of the present invention should have the property of N/C = 2 or greater, in other words, the LD50 value for healthy cells should be at least twice the LD50 value for cancer cells. Preferably, the LD50 with respect to healthy cells should be at least 10 times that of the LD50 with respect to cancer cells (N/C = 10 or greater), more preferably, N/C = 10-25, and subsequently, the range of preferability goes sequentially as N/C = 50, N/C = 50 or more, N/C = 50-100, N/C = 100 or more, N/C = 100-1000, even more preferably, N/C = 250-1000, and most preferably, N/C = 1000 or greater.

Pharmaceutical Composition

A formulation containing the human anti-TRAIL-R1 and R2 antibodies of the present invention is also included within the scope of the present invention. Such formulations preferably contain, in addition to the antibody, a physiologically acceptable diluent or carrier, and may be a mixture with other antibodies or other pharmaceuticals such as antibiotics. While a suitable carrier could contain a physiological saline solution, phosphate-buffered physiological saline solution, phosphate-buffered physiological saline glucose solution and buffered saline solution, there is no restriction to these. Alternatively, the antibody can be freeze-dried and used when needed by reconstitution by adding to a buffer solution as described above. The relevant preventive or therapeutic agent can be administered in various forms, example of which include oral delivery such as in tablet, capsule, granule, powder or syrup form, and non-oral delivery such as by injection, infusion or suppository.

While the dosage will differ according to symptoms, age and weight, an adult would normally take about 0.01-1000 mg once or a few times daily by oral delivery. Additionally, in the case of non-oral delivery, about 0.01-1000 mg could be delivered with each dose by hypodermic injection, intramuscular injection or intravenous injection.

The antibody or pharmaceutical composition of the present invention can be applied to the treatment or prevention of various diseases and symptoms which may be caused by cells expressing TRAIL-R1 and R2. Examples of such diseases or symptoms would include various types of malignant tumors.

Types of tumors include colon cancer, colorectal cancer, lung cancer, mammary cancer, brain tumors, melanoma, renal cell cancer, cystic cancer, leukemia, lymphoma, T-cell lymphoma, multiple myeloma, gastric cancer, pancreatic cancer, cervical cancer, endometrial cancer, ovarian cancer, esophagal cancer, hepatic cancer, squamous cancer, skin cancer, urethral cancer, prostate

cancer, chorionic cancer, pharyngeal cancer, laryngeal cancer, pleural cancer, androblastoma, endometrial hyperplasia, endometriosis, embryoma, fibrosarcoma, Kaposi sarcoma, hemangioma, cavernous hemangioma, angioblastoma, retinoblastoma, astrocytoma, neurofibroma, oligodendroglioma, medulloblastoma, neuroblastoma, neuroglioma, rhabdomyosarcoma, glioblastoma, osteogenic sarcoma, leiomyosarcoma, goiter and Wilms tumors, and the antibody of the present invention can be applied not only to a single type of tumor, but to multiple types of tumors which have occurred in combination.

Formulation Example

The molecules of the present invention is provided for use in the form of an ampule of a sterile solution or suspension in which they have been dissolved into water or another pharmacologically acceptable solution. Alternatively, the formulation can be loaded into an ampule in the form of a sterile powder (preferably the molecules of the present invention in freeze-dried form), which is diluted with a pharmacologically acceptable solution at the time of use.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the apoptosis-inducing activity of a hybridoma producing a human anti-TRAIL-R1 monoclonal antibody with respect to Colo205 in a conditioned medium.

Fig. 2 is a graph showing the apoptosis-inducing activity of a hybridoma producing a human anti-TRAIL-R2 monoclonal antibody with respect to Colo205 in a conditioned medium.

Fig. 3 is a graph showing the apoptosis-inducing activity of a hybridoma producing a human anti-TRAIL-R2 monoclonal antibody with respect to Colo205 in a conditioned medium (caprine anti-human IgG(γ) specific polyclonal antibody not added).

Fig. 4 is a graph showing the apoptosis-inducing activity of a hybridoma producing a human anti-TRAIL-R2 monoclonal antibody with respect to HUVEC in a conditioned medium.

Fig. 5a is a graph showing the apoptosis-inducing activity of recombinant human TRAIL (positive control) on Colo205 and healthy human hepatocytes.

Fig. 5b is a graph showing the apoptosis-inducing activity of a purified human anti-TRAIL-R2 monoclonal antibody (H-48-2) on Colo205 and healthy human hepatocytes.

Fig. 5c is a graph showing the apoptosis-inducing activity of a purified human anti-TRAIL-R2 monoclonal antibody (E-11-13) on Colo205 and healthy human hepatocytes.

Fig. 5d is a graph showing the apoptosis-inducing activity of a purified human anti-TRAIL-R2 monoclonal antibody (L-30-10) on Colo205 and healthy human hepatocytes.

Fig. 5e is a graph showing the apoptosis-inducing activity of a purified human anti-TRAIL-R2 monoclonal antibody (F-4-8) on Colo205 and healthy human hepatocytes.

Fig. 5f is a graph showing the apoptosis-inducing activity of a purified human anti-TRAIL-R2 monoclonal antibody (W-40-5) on Colo205 and healthy human hepatocytes.

Fig. 5g is a graph showing the apoptosis-inducing activity of a purified human anti-TRAIL-R2 monoclonal antibody (0304) on Colo205 and healthy human hepatocytes.

Fig. 5h is a graph showing the apoptosis-inducing activity of a purified human anti-TRAIL-R2 monoclonal antibody (0322) on Colo205 and healthy human hepatocytes.

Fig. 5i is a graph showing the apoptosis-inducing activity of a purified human anti-TRAIL-R2 monoclonal antibody (KMTR1) on Colo205 and healthy human hepatocytes.

Fig. 5j is a graph showing the apoptosis-inducing activity of a purified human anti-TRAIL-R2 monoclonal antibody (D1M) on Colo205 and healthy human hepatocytes.

Fig. 5k is a graph showing the apoptosis-inducing activity of a purified human anti-TRAIL-R2 monoclonal antibody (0304, caprine anti-human IgG antibody not added) on Colo205 and healthy human hepatocytes.

Fig. 5l is a graph showing the apoptosis-inducing activity of a purified human anti-TRAIL-R2 monoclonal antibody (KMTR1, caprine anti-human IgG antibody not added) on Colo205 and healthy human hepatocytes.

Fig. 6 is a graph showing the results of measurement of the size of a tumor after administering purified human anti-TRAIL-R2 monoclonal antibodies E-11-13, F-4-8, H-48-2, L-30-10 and W-40-5 at a dosage of 1 μg/mouse 3 times at one-day intervals.

Fig. 7 is a graph showing the results of measurement of the size of a tumor after administering purified human anti-TRAIL-R2 monoclonal antibody E-11-13 at dosages of 4, 20 and 100 μg/mouse 4 times.

Fig. 8 is a graph showing the results of measurement of the size of a tumor after administering purified human anti-TRAIL-R2 monoclonal antibody E-11-13 to 300 mm^3 cancerous mice at a dosage of 20 μg/mouse 3 times at one-day intervals.

Fig. 9 is a graph showing the results of measurement of the size of a tumor after administering purified human anti-TRAIL-R2 monoclonal antibody 0304 to 100 mm^3 cancerous mice at a dosage of 20 μg/mouse 3 times at one-day intervals.

Fig. 10 is a graph showing the results of measurement of the size of a tumor after administering purified human anti-TRAIL-R2 monoclonal antibody 0304 to 100 mm^3 cancerous mice at a dosage of 25 μg/mouse 3 times.

Fig. 11a is a graph showing the apoptosis-inducing activity (caprine anti-human IgG antibody not added) of recombinant purified human anti-TRAIL-R2 monoclonal antibodies with respect to Colo205 cells.

Fig. 11b is a graph showing the apoptosis-inducing activity (caprine anti-human IgG antibody not added) of recombinant purified human anti-TRAIL-R2 monoclonal antibodies with respect to Colo205 cells.

BEST MODES FOR CARRYING OUT THE INVENTION

Herebelow, the present invention shall be described in further detail by means of examples, but the present invention must not be construed as being restricted to only those modes described in the examples.

Example 1 Formulation of Antigen

In order to obtain cells excessively expressing human TRAIL-R1 and R2 at the cell membrane, plasmid vectors expressing human TRAIL-R1 and human TRAIL-R2 with the death domain in the intracellular region and the amino acids to the C-terminus side of the death domain removed from the full-length amino acid of human TRAIL-R1 and R2 (hereinafter referred to as "TRAIL-R1 and R2delta") were prepared. The DNA coding for TRAIL-R1 and R2delta was prepared by the PCR method.

a) Formulation of Full-Length Human TRAIL-R1 and R2 Expression Vectors

The templates used for performing template PCR were plasmid vectors pcDNA3-TRAIL-R1 and pcDNA3-TRAIL-R2 possessing cDNA coding for human TRAIL-R1 and R2. pcDNA3-TRAIL-R1 and pcDNA3-TRAIL-R2 were produced by the following methods. Full-length human TRAIL-R1 DNA and TRAIL-R2 DNA were modified by a polymerase chain reaction (PCR) so as to add an EcoRI sequence to the 5′ terminus and add a NotI sequence and a termination codon to the 3′ terminus. With human placental cDNA (Clontech) as the template, 5′-CACGAATTCACCATGGCGCCACCACCAGCT-3′ (Sequence No. 1) and 5′-TTTCTCGAGGCGGCCGCTTATCACTCCAAGGACACGGCAGAGCCTGTG-3′

(Sequence No. 2) were synthesized as primers for TRAIL-R1, and 5'-CACGAATTCGCCACCATGGAACAACGGGGACAG-3' (Sequence No. 3) and 5'-TTTCTCGAGGCGGCCGCTCATTAGGACATGGCAGAGTCTGCATTACCT-3' (Sequence No. 4) were synthesized as primers for TRAIL-R2, and platinum Pfx DNA polymerase (Gibco BRL) was used to perform a PCR reaction at (94 °C, 20 seconds; 60 °C, 30 seconds; 68 °C, 90 seconds) × 30 cycles. The modified TRAIL-R1 and TRAIL-R2 sequences were isolated as EcoRI-NotI fragments, and linked to pcDNA3 (Invitrogen) vectors, cleaved by the same enzyme. The resulting plasmids were named pcDNA3-TRAIL-R1 and pcDNA3-TRAIL-R2. The TRAIL-R2 integrated into the pcDNA3-TRAIL-R2 consisted of 440 amino acids coded by a 1320-bp cDNA among the two fragments formed by selective splicing. Herebelow, the reaction temperature control for all PCR reactions in the examples shall be performed using a GeneAmp PCR System 9700, product of Perkin-Elmer Japan.

b) Formulation of Human TRAIL-R1 and R2delta Expression Vectors

Human TRAIL-R1 and R2delta expression vectors were prepared by the following methods. In order to produce an expression plasmid comprising a TRAIL-R1 partial peptide having a 1-351 amino acid sequence and a TRAIL-R2 partial peptide having a 1-348 amino acid sequence, a PCR reaction was performed to add an EcoRI sequence to the 5' terminus and a NotI sequence and a termination codon to the 3' terminus of the TRAIL-R1 and R2 partial peptides. The PCR was performed with the oligonucleotide primers 5'-CACGAATTCACCATGGCGCCACCACCAGCT-3' (Sequence No. 1) and 5'-TTTCTCGAGGCGGCCGCTTATCACTCCAAGGACACGGCAGAGCCTGTG-3' (Sequence No. 2) for TRAIL-R1 and the oligonucleotide primers 5'-CACGAATTCGCCACCATGGAACAACGGGGACAG-3' (Sequence No. 3) and 5'-TTTCTCGAGGCGGCCGCTCATTAGGACATGGCAGAGTCTGCATTACCT-3' (Sequence No. 4) for TRAIL-R2, using a platinum Pfx DNA polymerase (Gibco BRL), and pcDNA3-TRAIL-R1 and pcDNA3-TRAIL-R2, under the conditions of (94 °C, 20 seconds; 65 °C, 30 seconds; 68 °C, 75 seconds) × 30 cycles. The modified TRAIL-R1 and R2 partial peptides were isolated as EcoRI-NotI fragments. Then, these EcoRI-NotI fragments were linked to pEFneo vectors cleaved with EcoRI and NotI enzymes. The resulting plasmids were named pEF-TRAIL-R1delta and pEF-TRAIL-R2delta.

c) Preparation of Human TRAIL-R1 and R2delta-Expressing Cells

The pEF-TRAIL-R1delta and pEF-TRAIL-R2delta prepared in b) were transfected into L929 cells (American Type Culture Collection No. CCL-1) using LipofectAMINE Plus (Gibco BRL). The transfection was performed according to the manual. After cultivating for 24 hours at 37 °C and in the presence of 5.0% carbon dioxide gas in a cell cultivating flask (culture are 75 cm^2), G418 (Gibco BRL) was added to a concentration of 1 mg/ml, and the result was further cultivated for 1 week. Then, a FACS analysis was performed using a caprine anti-human TRAIL-R1 polyclonal antibody and a caprine anti-human TRAIL-R2 polyclonal antibody (Dako), and those cells attaining the trait of G418 resistance were confirmed to express TRAIL-R1delta consisting of 351 amino acids and TRAIL-R2delta consisting of 348 amino acids at the surface of the cell membrane.

The oligonucleotides such as the primers for the PCR were all synthesized using a DNA autosynthesizer (Perkin-Elmer Japan, Applied Biosystems Division), in accordance with the manual [see Matteucci, M. D. and Caruthers, M. H. (1981) *J. Am. Chem. Soc.* 103, 3185-3191]. After synthesis, the oligonucleotides were cleaved from the support and deprotected, then the resulting solution was dried, dissolved in distilled water, and stored in freeze-dried form at -20 °C until use.

Example 2 Preparation of Human Antibody-Producing Mice

The mice used for immunization had a homozygotic genetic background for both intrinsic Ig heavy chain and κ light chain rupture, and possessed both a Chromosome 14 fragment (SC20) containing the human Ig heavy chain gene locus and the human Igκ chain transgene (KCo5). These mice were produced by mating mice of line A possessing the human Ig heavy chain gene locus with mice of line B possessing the human Igκ chain transgene. Line A was a strain of mice possessing the heritable Chromosome 14 fragment (SC20) which is a homozygote of both intrinsic Ig heavy chain and κ light chain rupture, such as is reported by Tomizuka *et al.* (Tomizuka *et al., Proc Natl Acad Sci USA*, 2000, vol. 97:722). Line B was a strain of mice (transgenic mice) possessing the human Igκ chain transgene (KCo5) which is a homozygote of both intrinsic Ig heavy chain and κ light chain rupture, such as is described in a report by Fishwild *et al.* (*Nat Biotechnol*, 1996, vol. 14:845).

The offspring of a male mouse of line A and a female mouse of line B, or of a female mouse of line A and a male mouse of line B were analyzed by the method reported by

Tomizuka *et al.* (Tomizuka *et al., Proc Natl Acad Sci USA,* 2000, vol. 97:722), and those exhibiting positive assays for both the human Ig heavy chain and κ light chain in the serum (human antibody-producing mice) were selected out (Ishida & Lonberg, *IBC's 11th Antibody Engineering,* Abstract 2000; Ishida, I. *et al., Cloning & Stem Cells* 4, 85-96 (2002)) for use in the following immunization experiments. Mice modified from the genetic background of the above-described mice (Ishida, I. (2002), *Jikken Igaku* 20, 6, 846-851). were also used in the immunization experiments. The above-described human antibody-producing mice are also available by contract from Kirin Brewery Co., Ltd.

Example 3 Preparation of Human Monoclonal Antibodies to Human TRAIL-R1 and R2

The monoclonal antibodies of the present example were prepared in accordance with generally accepted methods such as described in *Tan-clone Kotai Jikken Sosa Nyumon* (Ando, T. *et al.,* Kodansha, 1991). The human TRAIL-R1 and R2 to be used as immunogen was in the form of the TRAIL-R1 and R2delta expressing L929 cells produced in Example 1, or a protein fusing the extracellular region of human TRAIL-R1 and R2 with the Fc domain of human IgG1. The animals to be immunized were human antibody-producing mice producing human immunoglobulins as prepared in Example 2.

With the goal of producing a human monoclonal antibody to human TRAIL-R1, human antibody-producing mice were first immunized with TRAIL-R1delta-expressing L929 cells (3×10^6 cells/mouse) as produced in Example 1 through the right footpad. After the first immunization, they were immunized every three days with the same cells alternately in the left and right footpads for a total of 10 immunizations. They were further immunized with the same cells through both footpads three days prior to taking the spleen and lymph nodes as described below. With the goal of producing a human monoclonal antibody to human TRAIL-R2, human antibody-producing mice were first immunized with TRAIL-R1delta -expressing L929 cells (1×10^7 cells/mouse) as produced in Example 1 in the peritoneal cavity. After the first immunization, they were immunized weekly with the same cells in the peritoneal cavity for a total of 5 or 6 immunizations. They were further immunized with the same cells or with proteins fusing human TRAIL-R2 extracellular regions with human IgG1 Fc domains through the tail vein three days prior to taking the spleen as described below.. Additionally, human antibody-producing mice were first immunized subcutaneously using a mixture of proteins fusing human TRAIL-R2 extracellular regions with human IgG1 Fc domains and

complete Freund adjuvant, then they were immunized at two week intervals subcutaneously with a mixture of the same proteins and incomplete Freund adjuvant for a total of two immunizations, and immunized with the same proteins through the tail vein three days prior to taking the spleen as described below.

The spleen and/or lymph nodes were surgically excised from the immunized mice, put into 10 ml of a serum-free DMEM culture (Gibco BRL) containing 350 mg/ml of sodium bicarbonate, 50 units/ml of penicillin and 50 μg/ml of streptomycin (hereinafter referred to as "serum-free DMEM culture"), and crushed on a mesh (Cell Strainer, Falcon) using a spatula. A suspension of the cells passed through the mesh was centrifuged to precipitate the cells, these cells were rinsed twice with serum-free DMEM culture medium, and suspended in serum-free DMEM culture medium to measure the number of cells. On the other hand, myeloma cells SP2/0 (ATCC No. CRL-1581) cultivated in a DMEM culture medium (Gibco BRL) containing 10% FCS (Sigma) (hereinafter referred to as "DMEM culture medium with serum") at 37 °C in the presence of 5% carbon dioxide gas such that the cell concentration would not exceed 1×10^8 cells/ml were similarly rinsed in a serum-free DMEM culture medium, then suspended in a serum-free DMEM culture medium to measure the number of cells. A suspension of the recovered cells and a suspension of murine myeloma cells were mixed together at a cell number ratio of 5 : 1, and after centrifugation, the supernatant was completely removed. After slowly adding 1 ml of 50% (w/v) polyethylene glycol 1500 (Boehringer-Mannheim) as a fusing agent to a pellet thereof while stirring the pellet with the tip of a pipette, 1 ml of a serum-free DMEM culture medium preheated to 37 °C were slowly added in two steps, after which 7 ml of serum-free DMEM culture medium were further added. The fused cells obtained by removing the supernatant after centrifugation were provided for screening by means of the limiting dilution method described below. Hybridomas were selected by cultivating in a DMEM culture containing 10% fetal calf serum (FCS) together with hypoxanthine (H), aminopterin (A) and thymidine (T) (hereinafter referred to as "HAT", Sigma). Furthermore, a DMEM culture medium containing 10% FCS and HT (Sigma) was used to produce single clones by a limiting dilution method. The culture was made in a 96-hole microtiter plate (Becton Dickinson). The selection (screening) of hybridoma clones producing anti-human TRAIL-R1 and R2 human monoclonal antibodies and the characterization of human monoclonal antibodies produced by the respective hybridomas were performed by an enzyme-linked immunoabsorbent assay (ELISA) and a fluorescence-activated cell sorter (FACS), or alternatively by measuring the

apoptosis-inducing activity against cancer cells.

The ELISA method described in Examples 4 and 5, and the FACS method described in Example 6 were used to obtain multiple hybridomas having a human immunoglobulin γ chain (hIGγ) and a human immunoglobulin light chain κ, as well as human monoclonal antibodies reactive specifically to human TRAIL-R1 and/or R2. In each of the examples given below and including the present example as well as the tables and drawings provided as test results in the examples, each of the hybridoma clones producing the TRAIL-R1 and R2 monoclonal antibodies of the present invention are named by the use of symbols. Additionally, those having "antibody" added before or after the symbols refer to antibodies produce by the respective hybridomas, or to recombinant antibodies produced by host cells containing the antibody genes (full-length or variable region) isolated from the hybridomas. Additionally, there are cases in which the name of a hybridoma clone is represented by the name of an antibody, within such a range that does not sacrifice clarity of the text. The following hybridoma clones represent single clones: 1-13, 1-18, 1-32, 1-40, 1-43, 2-6, 2-11, 2-12, 2-18, 2-47, 2-52, 3-1, 3-7, 3-10, 3-23, 3-33, 3-42, 3-53, 1-13-6, 1-32-9, 1-40-4, 1-43-43, 2-6-48, 2-11-5, 2-12-10, 2-47-11, 2-52-12, 3-10-19, 3-23-8, 3-33-7, 3-42-3, 3-53-15, 2-18-2, 3-1-7, E-11, E-14, L-30, N-18, X-14, E-11-13, E-14-4, F-4-2, F-4-8, H-48-2, L-30-10, N-18-12, W-40-5, X-14-4, X-51-4, X-51-12, A-11, G-3, H-34, I-22, I-35, J-21, J-26, K-8, K-16, K-57, L-4, P-28, P-36, W-42, X-13, X-60, Z-23, 1-39, A-4-27, A-4-29, G-3-10, H-34-2, K-57-12, W-42-2, 0304, 0322, KMTR1 and D1M. Among these, H-48-2 was internationally deposited at the International Patent Organism Depositary at the National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan) dated May 18, 2001. The international deposit number is FERM BP-7599. Additionally, E-11-13, F-4-8 and L-30-10 were internationally deposited at the above-stated depository on August 8, 2001. Their international deposit number for E-11-13 is FERM BP-7698, that of F-4-8 is FERM BP-7699 and that of L-30-10 is FERM BP-7700. Additionally, E-11-13, F-4-8 and L-30-10 were internationally deposited at the above-stated depository on October 11, 2001. The international deposit number for E-11-13 is FERM BP-7770, that of F-4-8 is FERM BP-7768 and that of L-30-10 is FERM BP-7769. Additionally, 0304 and KMTR1 were internationally deposited at the above depository on May 10, 2002. The international deposit number for 0304 is FERM BP-8037 and that of KMTR1 is FERM BP-8038.

Example 4 Assay for Human Anti-TRAIL-R1 Monoclonal Antibody and Human Anti-TRAIL-R2 Monoclonal Antibody Having Human Immunoglobulin Light Chain κ (Igκ)

50 μl each of proteins fusing the extracellular region of human TRAIL-R1 and R2 with the Fc domain of human IgG1 (hereinafter referred to respectively as "TRAIL-R1-hFc" and "TRAIL-R2-hFc" (Alexis; with regard to TRAIL-R2-hFc, those consisting of 1-183 amino acids were also used as the extracellular region of TRAIL-R2)) in 0.5 μg/ml of phosphate-buffered physiological saline (hereinafter referred to as"PBS") were added to the respective wells of a 96-hole ELISA microplate (Maxisorp, Nunc) and incubated at room temperature for 1 hour or at 4 °C overnight to bind the TRAIL-R1-hFc or TRAIL-R2-hFc to a microplate. Next, the supernatant was discarded, blocking reagent (SuperBlock® Blocking Buffer, Pierce) was added to the respective wells and the result was incubated at room temperature for 30 minutes to block the portions of the TRAIL-R1-hFc or TRAIL-R2-hFc which were not bound. In this way, a microplate with each well coated with TRAIL-R1-hFc or TRAIL-R2-hFc was prepared.

The conditioned media (50 μl) of respective hybridomas were added to the wells which were then reacted for 1 hour at room temperature, and each well was rinsed twice with PBS containing 0.1% Tween 20 (PBS-T). Next, 50 μl of a solution of caprine anti-human Igκ antibody (50 μl/well, Biosource International) labeled with horseradish peroxidase diluted to 2000 times with PBS-T containing 10% Block Ace (Dainippon Pharmaceutical) was added to each well and incubated for 30 minutes at room temperature. The microplate was then rinsed three times with PBS-T, after which 100 μl of TMB color substrate fluid (Dako) were added to each well, and the result incubated for 20 minutes at room temperature. 0.5M sulfuric acid was then added to each well (100 μl/well) to stop the reaction. The absorption at a wavelength of 450 nm (reference wavelength 570 nm) was measured using a microplate reader (MTF-300, Corona Electric). Additionally, antibodies produced by the hybridomas 0304, 0322, KMTR1 and D1M were put to the above-described experiment using purified antibodies obtained using the methods described in Example 10.

The properties of a portion of the antibodies among the resulting anti-human TRAIL-R1 and R2 antibodies are shown in Table 1 and Table 2. Table 1 is a table showing the subclasses and crossreactivities of the human anti-TRAIL-R1 monoclonal antibodies obtained in this way. Table 2 is a table showing the subclasses and crossreactivities of the human anti-TRAIL-R2 monoclonal antibodies obtained in this way.

Table 1

Human Anti-TRAIL-R1 Antibody	Subclass	Crossreactivity	
		TRAIL-R1	TRAIL-R2
1-13	IgG4	+	-
1-18	IgG4	+	-
1-32	IgG1	+	-
1-40	IgG1	+	-
1-43	IgG1	+	-
2-6	IgG1	+	-
2-11	IgG1	+	-
2-12	IgG1	+	-
2-18	IgM	+	-
2-47	IgG4	+	-
2-52	IgG1	+	-
3-1	IgM	+	-
3-7	IgM	+	-
3-10	IgG4	+	-
3-23	IgG4	+	-
3-33	IgG4	+	-
3-42	IgG2	+	-
3-53	IgG1	+	-

+: reactive
-: non-reactive

Table 2

Human Anti-TRAIL-R2 Antibody	Subclass	Crossreactivity	
		TRAIL-R1	TRAIL-R2
A-4-27	IgM	-	+
A-4-29	IgM	+	+
A-11	IgM	-	+
E-11	IgG1	-	+
E-14	IgG1	-	+
F-4-2	IgG4	-	+
F-4-8	IgG1	-	+
G-3	IgM	-	+
H-34	IgM	-	+
H-48-2	IgG1	-	+
I-22	IgM	-	+
I-35	IgM	-	+
J-21	IgM	-	+
J-26	IgM	-	+
K-8	IgM	-	+
K-16	IgM	-	+
K-57	IgM	-	+
L-4	IgM	-	+
L-30	IgG1	-	+
N-18	IgG4	-	+
P-28	IgM	-	+
P-36	IgM	-	+
W-40-5	IfG1	-	+
W-42	IgM	-	+
X-13	IgM	-	+
X-14	IgG4	-	+
X-51-4	IgG1	-	+
X-51-12	IgG4	-	+
X-60	IgM	-	+
Z-23	IgM	-	+
1-39	IgM	-	+
0304	IgG4	-	+
0322	IgG4	-	+
KMTR1	IgG1	+	+
D1M	IgG1	+	+

+: reactive
-: non-reactive

Example 5 Identification of Monoclonal Antibody Subclasses

A microplate having wells coated with TRAIL-R1-hFc or TRAIL-R2-hFc was prepared by a method similar to that of Example 4, and each well was rinsed twice with PBS-T. Conditioned media (50 μl) of the respective hybridomas obtained in Example 4 were added to the wells of the microplate coated with TRAIL-R1-hFc or TRAIL-R2-hFc, and after reacting for 1 hour, each well was rinsed twice with PBS-T. Then, ovine anti-human IgG1 antibody, ovine anti-human IgG2 antibody, ovine anti-human IgG3 antibody or ovine anti-human IgG4 antibody (each diluted to 2000 times, 50 μl/well, The Binding Site) labeled with horseradish peroxidase was added to each well, and incubated for 1 hour at room temperature. After rinsing three times with PBS-T, a substrate buffer solution (TMB, 100 μl/well, Dako) was added to each well, and incubated for 20 minutes at room temperature. Next, 0.5M sulfuric acid (100 μl/well) was added to stop the reaction. The absorption at a wavelength of 450 nm (reference wavelength 570 nm) was measured with a microplate reader (MTP-300, Corona Electric). Additionally, the antibodies produced by the hybridomas 0304, 0322, KMTR1 and D1M were put to the above-described experiments by means of the resulting purified antibody using the method described in Example 10. The results are shown in Table 1 and Table 2.

Example 6 Reaction Test of Monoclonal Antibodies to TRAIL-R1 and R2-Expressing Cells

A study of the reactivity of each monoclonal antibody acquired in Example 4 with respect to the TRAIL-R1delta-expressing L929 cells and TRAIL-R2delta-expressing L929 cells produced in Example 1 was performed by FACS analysis. L929 cells, TRAIL-R1delta-expressing L929 cells and TRAIL-R2delta-expressing L929 cells were suspended at a concentration of 2×10^6/ml in a Staining Buffer (SB) of PBS containing 0.1% NaN_3 and 1% FCS including 1% rabbit serum. The cell suspension (100 μl/well) was injected into a 96-hole round--bottomed plate (Becton-Dickinson). After centrifugation (200 rpm, 4 °C, 2 minutes), the supernatant was discarded, and the conditioned media (50 μl) of the hybridomas cultivated in Example 3 were added and stirred, then let stand for 30 minutes at a temperature below freezing, then centrifuged (200 rpm, 4 °C, 2 minutes) to remove the supernatant. After rinsing the pellets twice with SB at 100 μl/well, 30 μl of a 0.0125 mg/ml RPE fluorescence-labeled rabbit anti-human Igκ $F(ab')_2$ antibody (Dako), then incubated for 30 minutes at a temperature below freezing. After rinsing twice with SB, the result was suspended in 300 μl of SB, and the fluorescence of each cell measured using a FACS (FACScan, Becton Dickinson). As a result, all

antibodies exhibited strong binding activity to only TRAIL-R1delta-expressing L929 cells or TRAIL-R2delta-expressing L929 cells, while no binding activity to the L929 cells was observed, thus indicating that the antibodies bind specifically to TRAIL-R1 and TRAIL-R2.

Example 7 Apoptosis-Inducing Activity in Cancer Cells

The conditioned media of the hybridomas producing human anti-TRAIL-R1 monoclonal antibodies or human anti-TRAIL-R2 monoclonal antibodies obtained in Example 3 and Examples 4-6 were used to measure the apoptosis-inducing activity with respect to colon cancer cells Colo205 (ATCC No. CCL-222). Colo205 cells cultivated in an RPMI culture medium containing 10% FCS were adjusted to a concentration of 2.5×10^4/ml, and 100 μl was injected into each well of a 96-hole flat-bottom plate (Becton Dickinson). After cultivating for 24 hours at 37 °C in the presence of 5.0% carbon dioxide gas, 50 μl/well of the conditioned media of the hybridomas were added, and in the case where the human anti-TRAIL-R1 monoclonal antibody or human anti-TRAIL-R2 monoclonal antibody was IgG, 10 μl of a caprine anti-human IgG(γ)-specific polyclonal antibody (Sigma) was added to each well such as to make the final concentration 5 μg/ml. With respect to a portion of the obtained hybridomas, wells to which caprine anti-human IgG(γ)-specific polyclonal antibody was not added were also prepared. As a positive control, a human recombinant TRAIL protein (Dako) was used at a final concentration of 100 ng/ml. Human IgG (Biogenesis) was used as a negative control. After cultivating for 48 hours at 37 °C in the presence of 5.0% carbon dioxide gas, MTS reagent (Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega) was prepared in accordance with the method suggested in the manual, and 20 μl were added to each well. After cultivating for another 2 hours at 37 °C in the presence of 5.0% carbon dioxide gas, the absorption at a wavelength of 490 nm (reference wavelength 630 nm) was measured with a microplate reader (1420 ARVO Multilabel Counter, Wallac), and the cell survival rate was computed using the mitochondrial reduction rate as an indicator. The survival rate of the cells in each well were computed according to the following formula: Survival Rate (%) = $100 \times (a - b)/(c - b)$ (where a denotes the measurement value for the relevant well, b denotes the measurement value for a cell-free well and c denotes the measurement value for a negative control well). The results are shown in Figs. 1-3 and Tables 3 and 4. Table 3 is a table indicating the apoptosis-inducing activity with respect to Colo205 and human hepatocytes in conditioned media of hybridomas producing human anti-TRAIL-R1 monoclonal antibodies,

and Table 4 is a table indicating the apoptosis-inducing activity with respect to Colo205 and human hepatocytes in conditioned media of hybridomas producing human anti-TRAIL-R2 monoclonal antibodies.

Table 3

Human Anti-TRAIL-R1 Antibody	Subclass	Human Hepatocyte Survival Rate	Colo205 Cell Survival Rate
1-13-6	IgG4	-	-
1-32-9	IgG1	-	-
1-40-4	IgG1	-	-
1-43-43	IgG1	-	-
2-6-48	IgG1	-	-
2-11-5	IgG1	++	++
2-12-10	IgG1	-	-
2-47-11	IgG4	+	+
2-52-12	IgG1	++	++
3-10-19	IgG4	-	-
3-23-8	IgG4	-	-
3-33-7	IgG4	-	-
3-42-3	IgG2	-	-
3-53-15	IgG1	-	-
2-18-2	IgM	++	++
3-1-7	IgM	-	+
sTRAIL 1 μg/ml	-	-	-

++: Survival Rate 80% or more
+: Survival Rate 21-79%
-: Survival Rate 20% or less

Table 4

Human Anti-TRAIL-R2 Antibody	Subclass	Human Hepatocyte Survival Rate	Colo205 Cell Survival Rate
E-11-13	IgG1	++	-
E-14-4	IgG1	+	+
F-4-2	IgG4	+	-
F-4-8	IgG1	-	-
H-48-2	IgG1	++	-
L-3-10	IgG1	++	-
N-18-12	IgG4	++	-
W-40-5	IgG1	++	+
X-14-4	IgG4	++	+
X-51-4	IgG1	-	-
X-51-12	IgG4	++	-
A-4-29	IgM	-	-
G-3-10	IgM	++	-
H-34-2	IgM	-	-
K-57-12	IgM	+	-
W-42-2	IgM	-	-
sTRAIL 1 μg/ml	-	-	-

++: Survival Rate 80% or more
+: Survival Rate 21-79%
-: Survival Rate 20% or less

The results show that the human anti-TRAIL-R1 and R2 monoclonal antibodies clearly have activity inducing apoptosis in Colo205 as compared with the negative control. Moreover, they show that among the portion of the human anti-TRAIL-R2 monoclonal antibodies which are IgG's, activity inducing apoptosis was exhibited even in the absence of a caprine anti-human IgG(γ)-specific polyclonal antibody (without crosslinking of human anti-TRAIL-R2 monoclonal antibodies).

Example 8 Apoptosis-Inducing Activity in Healthy Cells

The conditioned media of the hybridomas producing human anti-TRAIL-R2 monoclonal antibodies obtained in Examples 4-6 were used to measure the apoptosis-inducing activity with respect to healthy human umbilical vein endothelial cells HUVEC (Biowhittaker). HUVEC cells cultivated in an EGM-2 culture medium (Biowhittaker) were adjusted to a concentration of 5×10^4/ml, and 100 μl was injected into each well of a 96-hole flat-bottom plate (Becton Dickinson). After cultivating for 24 hours at 37 °C in the presence of 5.0% carbon dioxide gas, 50 μl/well of the conditioned media of the hybridomas were added, and in the case

where the human anti-TRAIL-R1 monoclonal antibody or human anti-TRAIL-R2 monoclonal antibody was IgG, 10 μl of a caprine anti-human IgG(γ)-specific polyclonal antibody (Sigma) was added to each well such as to make the final concentration 5 μg/ml. Human IgG (Biogenesis) was used as a negative control. After cultivating for 48 hours at 37 °C in the presence of 5.0% carbon dioxide gas, MTS reagent (Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega) was prepared in accordance with the method suggested in the manual, and 20 μl were added to each well. After cultivating for another 2 hours at 37 °C in the presence of 5.0% carbon dioxide gas, the absorption at a wavelength of 490 nm (reference wavelength 630 nm) was measured with a microplate reader (1420 ARVO Multilabel Counter, Wallac), and the cell survival rate was computed using the mitochondrial reduction rate as an indicator. The cell survival rate of the cells was calculated using the same formula as in Example 7.

The results are shown in Fig. 4. The results for the human anti-TRAIL-R2 monoclonal antibody and the negative control are almost identical, thus indicating that the human anti-TRAIL-R2 monoclonal antibody does not damage HUVEC cells.

Example 9 Apoptosis-Inducing Activity in Healthy Human Hepatocytes

The conditioned media of the hybridomas producing the human anti-TRAIL-R1 and R2 monoclonal antibodies obtained in Examples 4-6 were used to measure the apoptosis-inducing activity in healthy human hepatocytes (hereinafter referred to as "HH cells") (Tissue Transformation Technologies). First, freeze-dried HH cells were thawed to 37 °C and adjusted to a concentration of 7.5×10^5/ml using a CM 5300 culture medium (Cedra), then 100 μl were injected into each well in a 96-hole flat-bottom plate (Becton Dickinson) coated with collagen type I. After cultivating for 4.5 hours at 37 °C in the presence of 5.0% carbon dioxide gas, the medium was exchanged. After further cultivating for 24 hours at 37 °C in the presence of 5.0% carbon dioxide gas, the medium was again exchanged. Subsequently, conditioned media of the hybridomas were added at 50 μl/well, and 10 μl of caprine anti-human IgG(γ)-specific polyclonal antibody (Sigma) were added to each well so as to make the final concentration 5 μg/ml. Human IgG (Biogenesis) was used as a negative control. After cultivating for 24 hours at 37 °C in the presence of 5.0% carbon dioxide gas, the morphological changes in the HH cells were observed under a microscope. As a result, there was hardly any difference between the results for the human anti-TRAIL-R2 monoclonal

antibody and the negative control, thus indicating that the human anti-TRAIL-R2 monoclonal antibody does not damage HH cells.

Example 10 Preparation of Antibodies

The human anti-TRAIL-R1 and R2 monoclonal antibodies were purified from the conditioned media of the hybridomas obtained in Example 4 and Examples 6-7 by the following method. The conditioned media containing the human anti-TRAIL-R1 and R2 monoclonal antibodies were affinity-purified with rmp Protein A (Amersham Pharmacia Biotech) and 0.8 × 40 cm columns (Biorad), using PBS as the adsorbent buffer solution and 0.02M glycine buffer solution (pH 3) as the eluent buffer solution. The elution fraction was adjusted to around pH 7.2 by adding 1M Tris (pH 9.0). The antibody solution prepared in this way was substituted with PBS using a dialysis membrane (10000cut, spectrum laboratories) and filter-sterilized with a membrane filter Millex-GV (Millipore) having a pore size of 0.22 μm to obtain purified human anti-TRAIL-R1 and R2 monoclonal antibodies. The concentrations of the purified antibodies were computed by measuring the absorption at 280 nm and taking 1 mg/ml as 1.4 OD.

The conditioned media containing human anit-TRAIL-R1 and R2 monoclonal antibodies were prepared by the following method. First, hybridomas producing the human anti-TRAIL-R1 and R2 monoclonal antibodies were conditioned to an eRDF culture medium (Kyokuto Seiyaku) containing 10 ng/ml recombinant human IL-6 (R&D Systems) and 10% Low IgG Fetal Bovine Serum (HyClone). Next, they were conditioned to an eRDF culture medium (Kyokuto Seiyaku) containing bovine insulin (5 μg/ml, Gibco BRL), human transferin (5μg/ml, Gibco BRL), ethanolamine (0.01 mM, Sigma), sodium selenite (2.5×10^{-5} mM, Sigma), 10 ng/ml recombinant human IL-6 (R&D Systems) and 1% Low IgG fetal bovie serum (HyClone) for the purpose of partial antibody purification. This was then cultivated in a flask and the supernatant was recovered at the point at which the live cell rate of the hybridomas became 90%. The recovered supernatant was put through 10 μm and 0.2 μm filters (German Science) to remove various types of waste such as hybridomas.

Example 11 Apoptosis-Inducing Activity of Purified Human Anti-TRAIL-R2 Monoclonal Antibodies in Cancer Cells and Healthy Human Hepatocytes

The purified human anti-TRAIL-R2 monoclonal antibodies obtained in Example 10 were used to measure the apoptosis-inducing activity in colon cancer cells Colo205 (ATCC No.

CCL-222). Colo205 cells cultivated in an RPMI culture medium containing 10% FCS were adjusted to a concentration of 2.5×10^4/ml, and 100 μl were injected into each well of a 96-hole flat-bottom plate (Becton Dickinson). After cultivating for 24 hours at 37 °C in the presence of 5.0% carbon dioxide gas, the purified antibodies were added at 10 μl/well so as to make the final concentration 10, 100 or 1000 ng/ml, and 10 μl of caprine anti-human IgG(γ)-specific polyclonal antibodies (Sigma) were added to each well such as to make the final concentration 10 μg/ml. For some of the obtained hybridomas, wells were prepared without adding any caprine anti-human IgG(γ)-specific polyclonal antibodies. As a positive control, human recombinant TRAIL proteins (R&D Systems) were used at final concentrations of 0.1, 1 and 10 ng/ml. Human anti-HAS antibody was used as a negative control. These were cultivated for 48 hours at 37 °C in the presence of 5.0% carbon dioxide gas to induce reactions between the antibodies and the cell surfaces. The volume of a single reaction system was 120 μl. With regard to 0304 and KMTR1, experiments in which the crosslinker caprine anti-human IgG(γ)-specific monoclonal antibodies were not added (indicated as "alone" in Table 5) were also performed. In this case, the volume of each reaction system was 110 μl. After cultivation, an MTS reagent (Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega) was prepared in accordance with the method given in the instruction manual, and 20 μl were added to each well. After further cultivation for 2 hours at 37 °C in the presence of 5.0% carbon dioxide gas, the absorption at a wavelength of 490 nm (reference wavelength 630 nm) was measured with a microplate reader (1420 ARVO multilabel counter , Wallac), and the cell survival rate was computed using the mitochondrial reduction rate as an indicator. The cell survival rate for each well was calculated according to the same formula as in Example 7.

Next, the human anti-TRAIL-R2 monoclonal antibodies obtained in Example 10 were measured for their apoptosis-inducing activity in HH cells (Tissue Transformation Technologies, In Vitro Technologies). First, the freeze-dried HH cells were thawed to 37 °C and adjusted to a concentration of 7.5×10^5/ml using a CM5300 culture medium (Cedra), after which 100 μl were injected into each well of a 96-hole flat-bottom plate (Becton Dickinson) coated with collagen type I. After cultivating for 4.5 hours at 37 °C in the presence of 5.0% carbon dioxide gas, the culture medium was exchanged. This was further cultivated for another 24 hours at 37 °C in the presence of 5.0% carbon dioxide gas, and the culture medium was changed to a serum-free medium (a DMEM culture medium (Sigma) containing insulin (20 μg/ml, Sigma), glucagons (7 ng/ml, Sigma), hydrocortisone (7.5 μg/ml, Sigma), and human EGF (20 ng/ml, Becton

Dickinson) or a CM5300 medium. Then, purified antibodies were added at 10 μl/well so as to make the final concentrations of the purified antibodies 0.1, 1 and 10 μg/ml, and 10 μl of caprine anti-human IgG(γ)-specific polyclonal antibodies (Sigma) were further added to each well so as to make the final concentration 10 or 100 μg/ml. For some of the obtained hybridomas, wells were prepared without adding any caprine anti-human IgG(γ)-specific polyclonal antibodies. Human anti-HAS antibody was used as a negative control. These were cultivated for 24 hours at 37 °C in the presence of 5.0% carbon dioxide gas to induce reactions between the antibodies and the cell surfaces. The volume of a single reaction system was 120 μl. With regard to 0304 and KMTR1, experiments in which the crosslinker caprine anti-human IgG(γ)-specific monoclonal antibodies were not added (indicated as "alone" in Table 5) were also performed. In this case, the volume of each reaction system was 110 μl. After cultivation, the HH cells were rinsed twice with PBS, and 100 μl of PBS was added to each well, after which 10 μl/well of Triton X-100 was added to make the final concentration 0.8%. After letting stand for 1 hour at 37 °C, the living HH cells were dissolved 50 μl/well of soluble liquid was transferred to a 96-hole flat-bottom plate to undergo an LDH assay. An LDH assay reagent (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega) was prepared in accordance with the method stated in the instruction manual, and 50 μl were added to each well. The plates were shielded and let stand for 30 minutes at room temperature, after which reaction stopper solution (1M acetic acid, Promega) was added at 50 μl/well, and the absorption at a wavelength of 492 nm was measured with a microplate reader (1430 ARVO multilabel counter, Wallac). The cell survival rate was computed using the LDH enzyme activity as an indicator. The cell survival rate for each cell was calculated by the same formula as Example 7.

Next, the computed survival rate was used to calculate the LD50 value by the following method. The survival rate at each antibody concentration was plotted on a graph with the vertical axis representing the calculated survival rate and the horizontal axis representing the concentration of the antibody added to the cells, and adjacent points were joined to form curves. The equations fitting these curves were determined by recursive computation, and the antibody concentrations corresponding to a survival rate of 50% were calculated from these equations to find the LD50 values.

The results are shown in Figs. 5a-l and Table 5. In Fig. 5, the black circles and solid lines (--●--) represent healthy human hepatocytes, and the diamonds and dashed lines (- -◊- -) represent Colo205 cells. Additionally, Figs. 5k and 5l show the experimental results for cases

in which no caprine anti-human IgG(γ)-specific polyclonal antibodies have been added. Table 5 is a table showing the apoptosis-inducing activity (LD50 value) of purified human anti-TRAIL-R2 monoclonal antibodies in colon cancer cells Colo205 and in healthy human hepatocytes. The LD50 value for colon cancer cells Colo205 is the LD50 value measured after inoculating 100 μl of culture medium in each hole of a 96-hole flat-bottom plate with 2.5×10^3 cells, adding purified human anti-TRAIL-R2 monoclonal antibodies to the cells the following day, and allowing the passage of 48 hours for the reaction time with the antibodies. The LD50 value for healthy human hepatocytes is the LD50 value measured after inoculating 100 μl of culture medium in each hole of a 96-hole flat-bottom plate with 7.5×10^4 healthy cells (human hepatocytes), adding purified human anti-TRAIL-R2 monoclonal antibodies to the healthy cells (human hepatocytes) the following day, and allowing the passage of 24 hours for the reaction time with the antibodies. The purified human anti-TRAIL-R2 monoclonal antibodies clearly are active to induce apoptosis in Colo205 cells as compared with the negative control. Furthermore, the toxicity with respect to human hepatocytes is clearly lower for purified human anti-TRAIL-R2 monoclonal antibodies E-11-13, L-30-10 and KMTR1 than for human recombinant TRAIL or purified antibody H-48-2.

Additionally, the results of Example 4 show that KMTR1 clearly binds to the receptors of both TRAIL-R1 and TRAIL-R2, and that this antibody can be expected to provide an apoptosis-inducing signal through both the TRAIL-R1 and TRAIL-R2 receptors.

Here, a survival rate of at least 50% was indicated when adding 10 μg/ml of L-30-10 to the hepatocytes, which indicates that the LD50 of L-30-10 is at least 10 μg/ml. The LD50 value as determined by recursive computations from a graph plotting the antibody concentration and the survival rate was found to be 24 μg/ml. Additionally, the survival rate at the time 0.1 μg/ml of F-4-8 was added to the hepatocytes was 50% or less, thus indicating that F-4-8 has an LD50 of 0.1 μg/ml or less. Upon calculating the LD50 value based on recursive calculations as with L-30-10, it was found to be 0.002 μg/ml. The LD50 values of KMTR1 and D1M were both confirmed to be at least 10 μg/ml. Additionally, when caprine anti-human IgG(γ)-specific polyclonal antibodies are not added (hereinafter referred to as "when alone"), the survival rate of hepatocytes never went below 50% even when adding KMTR1 with an antibody dosage of 100 μg/ml, thus confirming that the LD50 value when alone was at least 100 μg/ml.

Next, the LD50 for healthy human hepatocytes was compared with the LD50 for Colo205 cells by determining their ratio (N/C ratio). As a result, the purified antibody E-11-3

had an N/C = 25.45 (at least 10 times), D1M had an N/C = 67 or greater (at least 10 times), 0304 when alone had an N/C = 50 (at least 10 times), L-30-10 had an N/C = 240 (at least 100 times) and KMTR1 when alone had an N/C = at least 1000 times, thus indicating that all of the antibodies excelled in efficacy and safety (Table 5).

Table 5

Purified Human Anti-TRAIL-R2 Antibody	Healthy Human Hepatocyte LD50 (μg/ml)	Colo205 LD50 (μg/ml)	N/C Ratio
E-11-13	2.8	0.11	25.45
F-4-8	0.002	0.02	0.1
H-48-2	0.12	0.15	0.8
L-30-10	24	0.1	240
W-40-5	7.47	0.7	10.7
0304	0.002	0.02	0.1
0322	0.06	0.04	1.5
KMTR1	>10	0.04	>250
D1M	>10	0.15	>67
0304 (alone)	1	0.02	50
KMTR1 (alone)	>100	0.1	>1000
Human Recombinant TRAIL	0.25 ng/ml	2 ng/ml	0.125

Using a similar method, the apoptosis-inducing activity of the purified human anti-TRAIL-R2 monoclonal antibody was studied using U251 cells (from glioma, Riken Gene Bank No. RCB0461) and Jurkat cells (from T-cell lymphoma, Dainippon Pharmaceuticals). In the test for U251 cells, 100 μl of culture medium per hole in a 96-hole flat-bottom plate were inoculated with 1.0×10^4 cells, cultivated at 37 °C in the presence of 5% CO_2, and the antibodies were added the following day, the cell survival rate being measured after letting stand for 48 hours in the above-mentioned environment. In the test for Jurkat cells, 100 μl of culture medium per hole in a 96-hole flat-bottom plate were inoculated with 4.0×10^4 cells, the antibodies were added, and these were cultivated for 48 hours at 37 °C in the presence of 5% CO_2, after which the cell survival rate was measured.

E-11-13 had an LD50 of 0.3 for U251 cells and an LD50 of 0.1 for Jurkat cells.

L-30-10 had an LD50 of 0.17 for U251 cells and an LD50 of 0.13 for Jurkat cells.

H-48-2 had an LD50 of 0.24 for U251 cells and an LD50 of 0.07 for Jurkat cells.

F-4-8 had an LD50 of 0.03 for U251 cells and an LD50 of 0.004 for Jurkat cells.

W-40-5 had an LD50 of 1.0 for U251 cells and an LD50 of 0.48 for Jurkat cells.

With regard to the U251 cells, the assay was performed in a system in which a cisplatin injection solution (Nippon Kayaku) was added to a final concentration of 4 μg/ml simultaneously with the antibodies.

Example 12 Effect of Purified Human Anti-TRAIL-R2 Monoclonal Antibody on Cancerous Mouse Model

The effects fo the human anti-TRAIL-R2 monoclonal antibody obtained in Example 10 were studied using cancerous mouse models in accordance with the methods described below.

4-6 week old Balb/c nude mice (obtained from CLEA Japan) were implanted with 5×10^6 colon cancer cells Colo205 per mouse subcutaneously at a dorsal portion. One week to 10 days after the implantation, the size of the attached tumor was measured, and the cancerous mice were grouped together into groups of 5-7 having tumors with an average size of about 100 mm^3 to 300 mm^3. The cancerous mice were administered 1, 4, 20, 25 and 100 μg per mouse of the purified antibodies (dissolved in 200 μl of PBS) through the peritoneal cavity, and the sizes of the tumors were measured. As a negative control antibody, the same amount of a human anti-HAS antibody was also used.

The results of the above-described experiment are shown in Figs. 6-10. A regression effect was seen in the groups administered 1 μg per mouse of purified human anti-TRAIL-R2 monoclonal antibodies E-11-13, F-4-8, H-48-2, L-30-10 and W-40-5, and the anti-tumor effect became weaker in the order of E-11-13, L-30-10, F-4-8, W-40-5 (fig. 6). In Fig. 6, upon administration three times at intervals of one day, a growth inhibition and regression effect was observed for at least 13 days counting from the time of the first treatment (H-48-2 clones).

In the groups administered E-11-13 at 4, 20 and 100 μg per mouse, an anti-tumor effect was observed in all of the mice. The greatest tumor regression effect was observed at the dosage of 20 μg/mouse (Fig. 7). In Fig. 7, upon administration four times at intervals of one day, a growth inhibition and regression effect was observed for at least 11 days counting from the time of the first treatment. The changes over time of the tumor volumes in the groups administered 20 μg/mouse four times at one day intervals (administered 7 days, 9 days, 11 days and 13 days after implantation) were as follows.

2 days after the first treatment (corresponding to day 9 in Fig. 7), the average tumor volume was 109.5 mm^3.

4 days after the first treatment (corresponding to day 11 in Fig. 7), the average tumor

volume was 85.1 mm^3.

6 days after the first treatment (corresponding to day 13 in Fig. 7), the average tumor volume was 64.3 mm^3.

8 days after the first treatment (corresponding to day 15 in Fig. 7), the average tumor volume was 61.8 mm^3.

11 days after the first treatment (corresponding to day 18 in Fig. 7), the average tumor volume was 78.9 mm^3.

The tumor volume was about 85.1 mm^3 four days after beginning the treatment, which corresponds to a shrinkage of more than 14% of the tumor. This shrinkage was maintained even on the eleventh day of treatment, thus demonstrating that the antibodies of the present invention have a high anti-tumor effect.

Furthermore, upon administering 20 μl per mouse of E-11-13 to a group of 7 cancerous mice with an average of about 300 mm^3, significant tumor regression was observed (Fig. 8). In Fig. 8, upon administration three times at intervals of one day, a growth inhibition and regression effect was observed for at least 18 days counting from the time of the first treatment. The changes over time of the tumor volumes in the group administered 20 μg/mouse three times at one day intervals (administered 9 days, 11 days and 13 days later) were as follows.

2 days after the first treatment (corresponding to day 11 in Fig. 8), the average tumor volume was 246.9 mm^3.

4 days after the first treatment (corresponding to day 13 in Fig. 8), the average tumor volume was 181.8 mm^3.

5 days after the first treatment (corresponding to day 14 in Fig. 8), the average tumor volume was 146.2 mm^3.

6 days after the first treatment (corresponding to day 15 in Fig. 8), the average tumor volume was 110.8 mm^3.

9 days after the first treatment (corresponding to day 18 in Fig. 8), the average tumor volume was 57.5 mm^3.

11 days after the first treatment (corresponding to day 20 in Fig. 8), the average tumor volume was 81.3 mm^3.

13 days after the first treatment (corresponding to day 22 in Fig. 8), the average tumor volume was 108.1 mm^3.

15 days after the first treatment (corresponding to day 24 in Fig. 8), the average tumor

volume was 127.8 mm^3.

18 days after the first treatment (corresponding to day 27 in Fig. 8), the average tumor volume was 163.3 mm^3.

The tumor volume was about 181.8 mm^3 four days after commencing the treatment, corresponding to a shrinkage in the tumor of at least 39%. This shrinkage was maintained even in the eighteenth day of the treatment, thus demonstrating that the antibodies of the present invention have a high anti-tumor effect.

The activity of the 0304 antibody was evaluated as follows. 6 week old Balb/c nude mice (obtained from CLEA Japan) were implanted with 5×10^6 colon cancer cells Colo205 per mouse subcutaneously at a dorsal portion. 8 days after the implantation, the size of the attached tumor was measured, and cancerous mice having tumors with an average size of about 100 mm^3 were grouped together into a group of five. The cancerous mice were administered 20 μg per mouse of the purified antibodies (dissolved in 200 μl of PBS) through the peritoneal cavity, and the sizes of the tumors were measured. In the groups administered 0304 at 20 μg per mouse three times with intervals of one day (administered 8 days, 10 days and 12 days after implantation), an anti-tumor effect was observed in all of the mice (Fig. 9). The changes over time of the tumor volumes were as follows.

2 days after the first treatment (corresponding to day 10 in Fig. 9), the average tumor volume was 142.092 mm^3.

4 days after the first treatment (corresponding to day 10 in Fig. 9), the average tumor volume was 34.138 mm^3.

7 days after the first treatment (corresponding to day 15 in Fig. 9), the average tumor volume was 18.641 mm^3.

11 days after the first treatment (corresponding to day 19 in Fig. 9), the average tumor volume was 9.339 mm^3.

The tumor volume was about 34.138 mm^3 four days after commencing the treatment, corresponding to a shrinkage in the tumor of at least 65%. This shrinkage was maintained even in the eleventh day of the treatment, thus demonstrating that the antibodies of the present invention have a high anti-tumor effect.

Next, 12 week old Balb/c nude mice (obtained from CLEA Japan) were implanted with 5×10^6 colon cancer cells Colo205 per mouse subcutaneously at a dorsal portion. 10 days after

the implantation, the size of the attached tumor was measured, and cancerous mice having tumors with an average size of about 100 mm^3 were grouped together into a group of five. The cancerous mice were administered 25 μg per mouse of the purified antibodies (dissolved in 200 μl of PBS) through the peritoneal cavity, and the sizes of the tumors were measured. As a negative control antibody, the same amount of human anti-HAS antibody was used. In the groups administered 0304 at 25 μg per mouse three times with intervals of one day (administered 10 days, 13 days and 15 days after implantation), an anti-tumor effect was observed in all of the mice (Fig. 10). The changes over time of the tumor volumes were as follows.

3 days after the first treatment (corresponding to day 13 in Fig. 10), the average tumor volume was 54.626 mm^3.

5 days after the first treatment (corresponding to day 15 in Fig. 10), the average tumor volume was 32.357 mm^3.

8 days after the first treatment (corresponding to day 18 in Fig. 10), the average tumor volume was 15.895 mm^3.

12 days after the first treatment (corresponding to day 22 in Fig. 10), the average tumor volume was 14.377 mm^3.

15 days after the first treatment (corresponding to day 25 in Fig. 10), the average tumor volume was 26.654 mm^3.

19 days after the first treatment (corresponding to day 29 in Fig. 10), the average tumor volume was 27.565 mm^3.

25 days after the first treatment (corresponding to day 35 in Fig. 10), the average tumor volume was 30.802 mm^3.

29 days after the first treatment (corresponding to day 39 in Fig. 10), the average tumor volume was 27.092 mm^3.

12 days after the first treatment (corresponding to day 22 in Fig. 10), the tumor was found to have disappeared in three out of the five mice.

The tumor volume was about 54.626 mm^3 three days after commencing the treatment, corresponding to a shrinkage in the tumor of at least 45%. Furthermore, the tumor volume was about 32.357 mm^3 five days after commencing the treatment, corresponding to a shrinkage in the tumor of at least 65%. This shrinkage was maintained even 32 days after the treatment, and shrinkage of at least 65% was maintained until at least day 27. Thus, these antibodies of

the present invention have an extremely high anti-tumor effect.

Furthermore, in Fig. 10, a growth inhibition and regression effect was observed until at least day 32 counting from the first treatment.

It should be noted here that in Figs. 9 and 10, the term "vehicle" is used to indicate PBS (200 μl) used as a medium for dissolution for administering the antibodies.

As demonstrated by Example 11, 0304 and KMTR1 are antibodies which exhibit apoptosis-inducing activity even when the antibody is used alone, and 0304 was confirmed to have a remarkable anti-tumor effect even in cancerous mouse models as demonstrated in the present example. Such antibodies which exhibit apoptosis-inducing activity and anti-tumor activity even when alone can be expected to have the possibility of exhibiting anti-tumor activity regardless of the physiological condition (such as the type and number of immunocytes) of the patient who is to receive a preventive or therapeutic agent, particularly a drug for treating malignant tumors, for a disease caused by cells expressing TRAIL-R1 and/or TRAIL-R2.

Example 13 Binding Affinity to TRAIL-R1 and TRAIL-R2 of Purified Human Anti-TRAIL-R1 and TRAIL-R2 Monoclonal Antibodies

The binding affinities to TRAIL-R of the purified human anti-TRAIL-R monoclonal antibodies obtained in Example 10 were analyze using a Biacore 2000 (Biacore) in accordance with the following method.

1) Immobilization of TRAIL-R1-hFc and TRAIL-R2-hFc

TRAIL-R1-hFc or TRAIL-R2-hFc was diluted with 10 mM acetic acid (pH 4.0) to a final concentration of 10 μg/ml, and immobilized on a sensor chip CM5 by the amine coupling method. The immobilization conditions were as follows, and the NHS activation and ethanol amine blocking were performed in accordance with the methods described in the instruction manual. TRAIL-R1-hFc and TRAIL-R2-hFc were coupled by means of manual injection as described in the instruction manual.

(Immobilization Conditions)

Flow Rate:	5 μl/minute
NHS Activation:	7 minutes
Coupling:	manual injection

Ethanol Amine Blocking: 7 minutes

With the above-described conditions, 377.4RU of TRAIL-R1-hFc and 495.4RU of TRAIL-R2-hFc were confirmed to be immobilized on the sensor chip.

2) Regeneration Conditions and Reproducibility Check

20 μg/ml purified human anti-TRAIL-R1 monoclonal antibody 2-6 was added over a period of 2 minutes to a sensor chip on which TRAIL-R1-hFc was immobilized, and the antibody was confirmed to have bound with TRAIL-R1-hFc. Then, 50 mM NaOH was added for 15 seconds, and the bound antibody was confirmed to have completely dissociated from the TRAIL-R1-hFc (herebelow, complete dissociation shall be referred to as "regeneration"). Next, purified human anti-TRAIL-R1 monoclonal antibody 2-6 was added to the regenerated TRAIL-R1-hFc at a flow rate of 20 μl/minute using the KINJECT method (1 minute bound, 1 minute dissociated), after which 50 mM NaOH was added for 15 seconds to regenerate TRAIL-R1-hFc, this cycle being repeated 9 times. Even after 9 repetitions of this cycle, no changes in the amount of TRAIL-R1-hFc immobilized on the sensor chip and amount of antibody bound were observed, thus making it clear that TRAIL-R1-hFc can be regenerated by adding 50 mM NaOH for 15 seconds, without being inactivated. A similar study was performed using a sensor chip on which TRAIL-R2-hFc was immobilized together with 20 μg/ml purified human anti-TRAIL-R2 monoclonal antibody E-11-13, confirming that TRAIL-R2-hFc can also be regenerated by the same regeneration conditions.

3) Interaction Analysis

Purified human anti-TRAIL-R1 monoclonal antibodies 1-13, 2-6 and 2-12 were step-diluted to 2.1, 4.2, 8.4, 16.8, 33.5, 7.0 and 134.0 nM using HBS-EP (Biacore), and the dilution series of each antibody was added sequentially at a flow rate of 20 μl/minute by the KINJECT method (2 minutes bound, 6 minutes dissociated) to obtain a sensorgram. Similarly, purified human anti-TRAIL-R2 monoclonal antibodies E-11-13, L-30-10, H-48-2, F-4-8, W-40-6 and X-14-4 were step-diluted to 0.52, 1.05, 2.1, 2.09, 4.19 and 8.38 nM using HBS-EP (Biacore), and the dilution series of each antibody was added sequentially at a flow rate of 20 μl/minute by the KINJECT method (2 minutes bound, 2 minutes dissociated) to obtain a sensorgram. For each antibody, the sensorgram was used to perform a kinetic analysis using BIAevaluation ver 3.2 (Biacore). A global fit was performed using a bivalent model as a fitting model to determine the association rate constant and the dissociation rate constant. Additionally, the dissociation constant (Kd) was calculated from these two constants. The sensorgrams used for the fit had

the control cell removed and were buffer-corrected. The results are shown in Tables 6 and 7. In the tables, *kass* denotes the association rate constant, *kdiss* denotes the dissociation rate constant, and K_D denotes the dissociation constant.

Table 6

Purified Human Anti-TRAIL-R1 Antibody	*kass* (l/Ms)	*kdiss* (l/s)	K_D (nM)
1-13	1.08×10^5	4.58×10^{-4}	4.24
2-6	1.62×10^5	1.86×10^{-4}	1.15
2-12	1.63×10^5	7.80×10^{-4}	4.79

Table 7

Purified Human Anti-TRAIL-R2 Antibody	*kass* (l/Ms)	*kdiss* (l/s)	K_D (nM)
E-11-13	5.27×10^5	3.84×10^{-4}	0.0729
L-30-10	6.13×10^5	1.44×10^{-4}	2.35
H-48-2	5.75×10^5	1.58×10^{-4}	2.75
F-4-8	5.63×10^5	7.05×10^{-4}	1.25
W-40-6	1.74×10^5	2.92×10^{-4}	16.8
X-14-4	6.55×10^5	2.93×10^{-4}	44.7

Example 14 Preparation of Genes Coding for Monoclonal Antibodies and Construction of Recombinant Antibody Expression Vectors

(1) cDNA Cloning of E-11-13, L-30-10 and H-48-2 Antibody Genes and Production of Expression Vectors

The hybridomas E-11-13, L-30-10 and H-48-2 were cultivated in an eRDF culture medium (Kyokuto Seiyaku) containing 10 ng/ml Recombinant Human IL-6 (R&D Systems) and 10% Low IgG Fetal Bovine Serum (HyClone) and the cells were collected by centrifugation, after which TRIZOL (Gibco BRL) was added and the total RNA were extracted in accordance with the instruction manual. The variable regions of the antibody cDNA were cloned using a SMART RACE cDNA Amplification Kit (Clontech) in accordance with the attached instruction manual.

A first strand of cDNA was prepared using 5 μg of the total RNA as a template.

1) Synthesis of First Strand cDNA

Total RNA	5 μg/3 μl
5′ CDS	1 μl
SMART oligo	1 μl

After incubating a reaction solution of the above-described composition for 2 minutes at 70 °C,

5 × Buffer	2 μl
DTT	1 μl
DNTP mix	1 μl
Superscript II	1 μl

were added and the result was incubated for 1.5 hours at 42 °C.

After further adding 100 μl of tricine buffer, the result was incubated for 7 minutes at 72 °C to obtain a first strand cDNA.

2) Amplification of Heavy-Chain Genes and Ligh-Chain Genes by PCR and Construction of Recombinant Antibody Expression Vector

Z-Taq available from Takara was used for amplification of the cDNA.

cDNA	2 μl
10 × Z-Taq Buffer	5 μl
dNTP mix	4 μl
Z-Taq	1 μl
Primer 1	
Primer 2	

A reaction solution of the above-described composition was diluted to a final volume of 50 μl using redistilled water, and provided to a PCR.

The heavy-chain amplification was performed using UMP (SMART RACE cDNA Amplification Kit, Clontech) and hh-6 primer (5′-GGT CCG GGA GAT CAT GAG GGT GTC CTT-3′) (Sequence No. 7) to repeat 30 cycles of 1 second at 98 °C and 30 seconds at 68 °C. Then, 1 μl of the reaction solution was taken as a template and NUMP (SMART RACE cDNA

Amplification Kit, Clontech) and hh-3 primer (5'-GTG CAC GCC GCT GGT CAG GGC GCC TG-3') (Sequence No. 8) were used to repeat 20 cycles of 1 second at 98 °C and 30 seconds at 68 °C. Subsequently, the amplified PCR product was purified by means of a PCR purification kit (Qiagen) and hh-4 (5'-GGT GCC AGG GGG AAG ACC GAT GG-3') (Sequence No. 9) was used as a primer to determine the base sequence. Since the sequence information revealed that the three clones E-11-13, L-30-10 and H-48-2 all have matching sequences at the N-terminus region, the same primer was used for subcloning and base sequence determination. Based on the sequence information, tnH48KBgl (5'-ATA TAG ATC TCT CAG TTA GGA CCC AGA GGG AAC C-3') (Sequence No. 10) was synthesized, and this primer was used to determine the sequence also from the opposite direction. A specific primer and tnCHNhe (5'-GAT GGG CCC TTG GTG CTA GCT GAG GAG ACG G-3') (Sequence No. 11) were used to perform PCR (1 second at 98 °C, 30 seconds at 60 °C, 30 seconds at 72 °C), the heavy-chain amplified cDNA fragments were consumed with SalI and NheI, and introduced into an N5KG1-Val Lark vector (IDEC Pharmaceuticals, modified vector of N5KG1 (US Patent No. 6,001,358)) cleaved by the same enzyme. The inserted sequence was confirmed to be the same as that determined by the direct sequence, by determining the sequence with the vector as template.

The light-chain amplification was performed using UMP (SMART RACE cDNA Amplification Kit, Clontech) and hk-2 (5'-GTT GAA GCT CTT TGT GAC GGG CGA GC-3') (Sequence No. 12) primer to repeat 30 cycles of 1 second at 98 °C and 30 seconds at 68 °C. Then, 1 μl of the reaction solution was taken as a template and NUMP (SMART RACE cDNA Amplification Kit, Clontech) and hk-6 (5'-T GGC GGG AAG ATG AAG ACA GAT GGT G-3') (Sequence No. 13) were used to repeat 20 cycles of 1 second at 98 °C and 30 seconds at 68 °C. Subsequently, the amplified PCR product was purified by means of a PCR purification kit (Qiagen) and hk-6 (5'-tggc ggg aag atg aag aca gat ggt g-3') primer was used to determine the base sequence. Since the sequence information revealed that the three clones all have matching sequences at the N-terminus region, the same primer was used for subcloning. Based on the sequence information, tnH48Hsal (5'-ATA TGT CGA CTA CGG GGG GGC TTT CTG AGA GTC-3') (Sequence No. 14) was synthesized, and this primer was used to determine the sequence also from the opposite direction. A specific primer and tnCkBsi (5'-AAG ACA GAT GGT GCA GCC ACC GTA CGT TTG AT-3') (Sequence No. 15) were used to perform PCR (1 second at 98 °C, 30 seconds at 60 °C, 30 seconds at 72 °C), the light-chain amplified cDNA fragments were consumed with BglII and BsiWI, and introduced into an N5KG1-Val Lark

vector cleaved by the same enzyme. The inserted sequence was confirmed to be the same as that determined by the direct sequence, by determining the sequence with the vector as template.

The DNA coding for the E-11-13 heavy chain variable domain and light chain variable domain, and the amino acid sequence of the heavy chain variable domain and light chain variable domain are as follows.

<E-11-13 Heavy Chain Variable Domain> (Sequence No. 16)

GTCGACTACGGGGGGGCTTTCTGAGAGTCATGGATCTCATGTGCAAGAAAATGAAGCACCTGTGGTTCTTCC
TCCTGCTGGTGGCGGCTCCCAGATGGGTCCTGTCCCAGCTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGA
AGCCTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGGCTCCATCATCAGTAAAAGTTCCTACTGGG
GCTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGAGTATCTATTATAGTGGGAGTACCTTCT
ACAACCCGTCCCTCAAGAGTCGAGTCACCATATCCGTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGA
GCTCTGTGACCGCCGCAGACACGGCTGTGTATTACTGTGCGAGACTGACAGTGGCTGAGTTTGACTACTGGG
GCCAGGGAACCCTGGTCACCGTCTCCTCAGCTAGC

<E-11-13 Heavy Chain Variable Domain> (Sequence No. 17)

MDLMCKKMKHLWFFLLLVAAPRWVLSQLQLQESGPGLVKPSETLSLTCTV
SGGSIISKSSYWGWIRQPPGKGLEWIGSIYYSGSTFYNPSLKSRVTISVDTSK
NQFSLKLSSVTAADTAVYYCARLTVAEFDYWGQGTLVTVSSAS

<E-11-13 Light Chain Variable Domain> (Sequence No. 18)

TCACAGATCTCTCAGTTAGGACCCAGAGGGAACCATGGAAGCCCCAGCTCAGCTTCTCTTCCTCCTGCTACT
CTGGCTCCCAGATACCACCGGAGAAATTGTGTTGACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGA
AAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCTTCTTAGCCTGGTACCAACAGAAACCTGG
CCAGGCTCCCAGGCTCCTCATCTATGATGCATCCAACAGGGCCACTGGCATCCCAGCCAGGTTCAGTGGCAG
TGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTACTGTCA
GCAGCGTAGCAACTGGCCTCTCACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGTACG

<E-11-13 Light Chain Variable Domain> (Sequence No. 19)

MEAPAQLLFLLLLWLPDTTGEIVLTQSPATLSLSPGERATLSCRASQSVSSFL
AWYQQKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVY
YCQQRSNWPLTFGPGTKVDIKRT

The translation starting point of the heavy chain DNA is the ATG codon beginning with adenine (A) at number 30 from the 5′ terminus of Sequence No. 16, with the boundary between the antibody variable domain and constant domain lying between the adenine (A) at number 461 and the guanine (G) at number 462 from the 5′ terminus. In the amino acid sequence, the heavy chain variable domain extends from the N terminus of Sequence No. 17 to the serine (S) residue at number 144, with the alanine (A) at number 145 marking the beginning of the constant domain. An analysis of the N terminus of the purified heavy chain protein showed that the heavy chain signal sequence extends from the N terminus of Sequence No. 17 to the serine (S) at number 26, and that the N terminus in the mature form is the glutamine (Q) at number 27 of Sequence No. 17.

The translation starting point of the light chain DNA is the ATG codon beginning with adenine (A) at number 35 from the 5′ terminus of Sequence No. 18, with the variable domain extending from the 5′ terminus to the adenine (A) at number 415. In the amino acid sequence, the variable domain extends from the N terminus of Sequence No. 19 to the lysine (K) at number 127. An analysis of the N terminus of the purified light chain protein showed that the light chain signal sequence extends from the N terminus of Sequence No. 19 to the glycine (G) at number 20, and that the N terminus in the mature form is the glutamic acid (E) at number 21 of Sequence No. 19.

The DNA coding for the L-30-10 heavy chain variable domain and light chain variable domain, and the amino acid sequence of the heavy chain variable domain and light chain variable domain are as follows.

<L-30-10 Heavy Chain Variable Domain> (Sequence No. 20)

GTCGACTACGGGGGGGCTTTCTGAGAGTCATGGATCTCATGTGCAAGAAAATGAAGCACCTGTGGTTCTTCC
TCCTGCTGGTGGCGGCTCCCAGATGGGTCCTGTCCCAGTTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGA
AGCCCTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTAGGAGTAACTACTGGG
GCTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGAATGTCTATTATAGAGGGAGCACCTACT
ACAATTCGTCCCTCAAGAGTCGAGTCACCATATCCGTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGA
GCTCTGTGACCGTCGCAGACACGGCTGTGTATTACTGTGCGAGACTGTCAGTGGCTGAGTTTGACTACTGGG
GCCAGGGAATCCTGGTCACCGTCTCCTCAGCTAGC

<L-30-10 Heavy Chain Variable Domain> (Sequence No. 21)

MDLMCKKMKHLWFFLLLVAAPRWVLSQLQLQESGPGLVKPSETLSLTCTV
SGGSISSRSNYWGWIRQPPGKGLEWIGNVYYRGSTYYNSSLKSRVTISVDTS
KNQFSLKLSSVTVADTAVYYCARLSVAEFDYWGQGILVTVSSAS

<L-30-10 Light Chain Variable Domain> (Sequence No. 22)

AGATCTCTCAGTTAGGACCCAGAGGGAACCATGGAAGCCCCAGCTCAGCTTCTCTTCCTCCTGCTACTCTGG
CTCCCAGATACCACCGGAGAAATTGTGTTGACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGA
GCCACCCTCTCTTGTAGGGCCAGTCAGAGTGTTAGCAGCTTCTTAGCCTGGTACCAACAGAAACCTGGCCAG
GCTCCCAGGCTCCTCATCTATGATGCATCCAACAGGGCCACTGGCAGCCCAGCCAGGTTCAGTGGCAGTGGG
TCTGGGACAGACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTACTGTCAGCAG
CGTAGCGACTGGCCTCTCACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGTACG

<L-30-10 Light Chain Variable Domain> (Sequence No. 23)

MEAPAQLLFLLLLWLPDTTGEIVLTQSPATLSLSPGERATLSCRASQSVSSFL
AWYQQKPGQAPRLLIYDASNRATGSPARFSGSGSGTDFTLTISSLEPEDFAV
YYCQQRSDWPLTFGPGTKVDIKRT

The translation starting point of the heavy chain DNA is the ATG codon beginning with adenine (A) at number 30 from the 5′ terminus of Sequence No. 20, with the boundary

between the antibody variable domain and constant domain lying between the adenine (A) at number 461 and the guanine (G) at number 462 from the 5′ terminus. In the amino acid sequence, the heavy chain variable domain extends from the N terminus of Sequence No. 21 to the serine (S) residue at number 144, with the alanine (A) at number 145 marking the beginning of the constant domain. The heavy chain signal sequence was estimated by a gene sequence prediction application (Signal P ver. 2) to extend from the N terminus of Sequence No. 21 to the serine (S) at number 26. An analysis of the N terminus of the purified heavy chain protein showed that the heavy chain signal sequence extends from the N terminus of Sequence No. 21 to the serine (S) at number 26, and that the N terminus in the mature form is the glutamine (Q) at number 27 of Sequence No. 21.

The translation starting point of the light chain DNA is the ATG codon beginning with adenine (A) at number 31 from the 5′ terminus of Sequence No. 22, with the variable domain extending from the 5′ terminus to the adenine (A) at number 411. In the amino acid sequence, the variable domain extends from the N terminus of Sequence No. 23 to the lysine (K) at number 127. An analysis of the N terminus of the purified light chain protein showed that the light chain signal sequence extends from the N terminus of Sequence No. 23 to the glycine (G) at number 20, and that the N terminus in the mature form is the glutamic acid (E) at number 21 of Sequence No. 23.

The DNA coding for the H-48-2 heavy chain variable domain and light chain variable domain, and the amino acid sequence of the heavy chain variable domain and light chain variable domain are as follows.

<H-48-2 Heavy Chain Variable Domain> (Sequence No. 24>

TCGACTACGGGGGGGCTTTCTGAGAGTCATGGATCTCATGTGCAAGAAAATGAAGCACCTGTGGTTCTTCCT
CCTGCTGGTGGCGGCTCCCAGATGGGTCCTGTCCCAGCTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAA
GCCTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTAGTAGTTACTACTGGGG
CTGGGTCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGAGTATCCATTATAGTGGGAGTACTTTCTA
CAACCCGTCCCTCAAGAGTCGAGTCACCATTTCCGTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGAG
CTCTGTGACCGCCGCAGACACGACTGTGTATTACTGTGCGAGACAGGGGTCTACTGTGGTTCGGGGAGTTTA
CTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGCTAGC

<H-48-2 Heavy Chain Variable Domain> (Sequence No. 25>

MDLMCKKMKHLWFFLLLVAAPRWVLSQLQLQESGPGLVKPSETLSLTCTV
SGGSISSSSYYWGWVRQPPGKGLEWIGSIHYSGSTFYNPSLKSRVTISVDTS
KNQFSLKLSSVTAADTTVYYCARQGSTVVRGVYYYGMDVWGQGTTVTVSS
AS

<H-48-2 Light Chain Variable Domain> (Sequence No. 26>

AGATCTCTCAGTTAGGACCCAGAGGGAACCATGGAAACCCCAGCGCAGCTTCTCTTCCTCCTGCTACTCTGG
CTCCCAGATACCACCGGAGAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGA
GCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGGTACCAGCAGAAACCTGGC
CAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGT
GGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAG
CAGTATGGTAGCTCACCTCTGTACACTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGTACG

<H-48-2 Light Chain Variable Domain> (Sequence No. 27>

METPAQLLFLLLLWLPDTTGEIVLTQSPGTLSLSPGERATLSCRASQSVSSS
YLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDF
AVYYCQQYGSSPLYTFGQGTKLEIKRT

The translation starting point of the heavy chain DNA is the ATG codon beginning with adenine (A) at number 29 from the 5′ terminus of Sequence No. 24, with the boundary between the antibody variable domain and constant domain lying between the adenine (A) at number 484 and the guanine (G) at number 485 from the 5′ terminus. In the amino acid sequence, the heavy chain variable domain extends from the N terminus of Sequence No. 25 to the serine (S) residue at number 152, with the alanine (A) at number 153 marking the beginning of the constant domain. The heavy chain signal sequence was estimated by a gene sequence prediction application (Signal P ver. 2) to extend from the N terminus of Sequence No. 25 to the serine (S) at number 26. An analysis of the N terminus of the purified heavy chain protein

showed that the heavy chain signal sequence extends from the N terminus of Sequence No. 25 to the serine (S) at number 26, and that the N terminus in the mature form is the glutamine (Q) at number 27 of Sequence No. 25.

The translation starting point of the light chain DNA is the ATG codon beginning with adenine (A) at number 31 from the 5' terminus of Sequence No. 26, with the variable domain extending from the 5' terminus to the adenine (A) at number 417. In the amino acid sequence, the variable domain extends from the N terminus of Sequence No. 27 to the lysine (K) at number 129. An analysis of the N terminus of the purified light chain protein showed that the light chain signal sequence extends from the N terminus of Sequence No. 27 to the glycine (G) at number 20, and that the N terminus in the mature form is the glutamic acid (E) at number 21 of Sequence No. 27.

(2) cDNA Cloning of 0304 Antibody Genes and Production of Expression Vectors

Hybridomas 0304 were collected by means of centrifugation, and about 900 μg of RNA were purified in accordance with protocol using the RNA extraction reagent ISOGEN (Nippon Gene). Next, 13 μg of polyA$^+$ RNA were obtained from 300 μg of the RNA using Oligotex™-dT30 <Super> (Takara Shuzo). The resulting polyA$^+$ RNA was used as the material in a SMART RACE cDNA Amplification Kit (Clontech Company), and a cloning experiment was performed in accordance with the attached instruction manual to obtain cDNA from an antibody gene variable domain. Specifically, first strand cDNA synthesis was performed by means of a reverse transcriptase with 1.0 μg of purified polyA$^+$ RNA as the raw material. The resulting cDNA was used as the template to amplify the H chain leader sequence and variable domain (hereinafter referred to also as "HV") and the L chain leader sequence and variable domain (hereinafter referred to also as "LV") by means of PCR using PCR primers specific to the DNA of heavy chain (hereinafter referred to also as "H chain") constant domain and light chain (hereinafter referred to also as "L chain") constant domain of human antibodies (IgGlp for the H chain, hk-2 for the L chain), and UMP primers provided with the SMART RACE cDNA Amplification Kit (oligonucleotides complementary to the common sequences formed on the 5' terminus of synthesized cDNA) as the primer set. For the PCR, TaKaRa LA Taq™ (Takara Shuzo), which is a Taq DNA polymerase for LA PCR, was used. The template DNA were added to a solution containing 400 μM (final concentration) each of 1 × LA PCR Buffer II (Mg^{2+} plus) and dNTP Mixture, 0.2 μM of two types of primer and TaKaRa LA Taq 2.5 U/50 μl, and

reacted by touchdown PCR (5 seconds at 94 °C and 3 minutes at 72 °C (5 cycles), 5 seconds at 94 °C, 10 seconds at 70 °C and 3 minutes at 72 °C (5 cycles), 5 seconds at 94 °C, 10 seconds at 68 °C, and 3 minutes at 72 °C (20 cycles)). After collecting the amplified PCR fragments by ethanol precipitation, they were recovered by agarose gel electrophoresis, then purified in a QIAquick Gel Extraction Kit (Qiagen) which is a DNA purifying kit. The DNA base sequences of the purified HV and LV fragments were determined used ABI PRISM® 3700 DNA Analyzer (Applied Biosystems). Additionally, the amplified HV and LV fragments were subcloned with pGEM®-T Easy Vector System (Promega) using a TA cloning method, and the base sequences of the inserted DNA in the plasmid DNA of the resulting clones were analyzed and compared with the direct sequence analysis results for the PCR product. The primer sequences used to determine the DNA base sequences (hh-4 for the H chain, hk-5 and hk-6 fo the L chain and SP6 and T7 for pGEM®-T Easy Vector) are shown in Table 8. The direct sequence analysis results for the PCR fragments of the HV and LV completely matched the DNA base sequence analysis results of a plurality of clones which were subcloned.

Using the DNA of the 0304 antibody L chain as the template, the leader sequence and variable domain of the L chain were amplified by PCR using a primer designed to add a restriction enzyme site for coupling at an end. The sequence of the primer set which was used is shown in Table 8 (C23LBCL and C23Lbsi). The resulting PCR fragments were collected by ethanol precipitation, then consumed by the restriction enzyme Bgl II, and further cleaved with BsiWI. A fragment of about 400 bp were recovered by agarose gel electrophoresis, and purified with a QIAquick Gel Extraction Kit (Qiagen) which is a DNA purifying kit using a membrane. On the other hand, the vector N5KG4-Val Lark (IDEC Pharmaceuticals, modified vector of N5KG1 (US Patent No. 6,001,358)) was similarly processed sequentially by the restriction enzymes Bgl II and BsiW I, and after processing with alkaline phosphatase (*E. coli* C75) (Takara Shuzo) as a dephosphorylation treatment, about 9 kb of DNA were recovered by agarose gel electrophoresis and a DNA purifying kit. These two fragments were ligated using T4 DNA ligase, and incorporated into E. coli DH5α for transformation. The plasmid DNA N5KG4-0304L, obtained by inserting a 0304 antibody L chain leader and a variable domain into N5KG4-Val Lark, was selected, and the DNA base sequence in the area around the inserted fragment was determined to confirm that there were no changes or the like in the DNA base sequence. In order to insert an H chain variable domain or the like into the N5KG4-0304L

obtained in this way, the present plasmid DNA was cleaved sequentially by the restriction enzymes Nhe I and Sal I, then dephosphorylated to purify a vector DNA of about 9.3 kb. On the other hand, the plasmid DNA of the antibody H chain was used as a template to amplify the leader sequence and variable domain of the 0304 antibody H chain gene by PCR. The sequence of the primer set (T0304Sal and T0304Nhe) used for amplification is shown in Table 8.

The resulting PCR fragments were cleaved by means of restriction enzymes Nhe I and Sal I, and fragments of about 450 bp were purified by agarose gel electrophoresis. These two types of DNA were ligated and introduced into *E. coli* to obtain transformations, and clones into which the desired H chain leader sequence and variable domain had been inserted were selected. The DNA base sequences of the inserted portions were determined, and the sequence inserted by PCR amplification was checked to make sure there were no differences from the gene sequence used as the template.

The DNA coding for the 0304 heavy chain variable domain and light chain variable domain, and the amino acid sequence of the heavy chain variable domain and light chain variable domain are as follows.

<0304 Heavy Chain Variable Domain> (Sequence No. 28)

CTCAACAACC ACATCTGTCC TCTAGAGAAA ACCCTGTGAG CACAGCTCCT CACCATGGAC
TGGACCTGGA GGATCCTCTT CTTGGTGGCA GCAGCTACAA GTGCCCACTC CCAGGTGCAG
CTGGTGCAGT CTGGGGCTGA GATGAAGAAG CCTGGGGCCT CAGTCAAGGT CTCCTGCAAG
ACTTCTGGAT ACACCTTCAC CAATTATAAG ATCAACTGGG TGCGACAGGC CCCTGGACAA
GGACTTGAGT GGATGGGATG GATGAACCCT GACACTGATA GCACAGGCTA TCCACAGAAG
TTCCAGGGCA GAGTCACCAT GACCAGGAAC ACCTCCATAA GCACAGCCTA CATGGAGCTG
AGCAGCCTGA GATCTGAGGA CACGGCCGTG TATTACTGTG CGAGATCCTA TGGTTCGGGG
AGTTATTATA GAGACTATTA CTACGGTATG GACGTCTGGG GCCAAGGGAC CACGGTCACC
GTCTCCTCA

<0304 Heavy Chain Variable Domain> (Sequence No. 29)

MDWTWRILFL VAAATSAHSQ VQLVQSGAEM KKPGASVKVS CKTSGYTFTN YKINWVRQAP
GQGLEWMGWM NPDTDSTGYP QKFQGRVTMT RNTSISTAYM ELSSLRSEDT AVYYCARSYG
SGSYYRDYYY GMDVWGQGTT VTVSS

<0304 Light Chain Variable Domain> (Sequence No. 30)

GAGGAACTGC TCAGTTAGGA CCCAGAGGGA ACCATGGAAG CCCCAGCTCA GCTTCTCTTC
CTCCTGCTAC TCTGGCTCCC AGATACCACC GGAGAAATTG TGTTGACACA GTCTCCAGCC
ACCCTGTCTT TGTCTCCAGG GGAAAGAGCC ACCCTCTCCT GCAGGGCCAG TCAGAGTGTT
AGCAGCTACT TAGCCTGGTA CCAACAGAAA CCTGGCCAGG CTCCCAGGCT CCTCATCTAT
GATGCATCCA ACAGGGCCAC TGGCATCCCA GCCAGGTTCA GTGGCAGTGG GTCTGGGACA
GACTTCACTC TCACCATCAG CAGCCTAGAG CCTGAAGATT TTGCAGTTTA TTACTGTCAG
CAGCGTAGCA ACTGGCCGCT CACTTTCGGC GGAGGGACCA AGGTGGAGAT CAAACGA

<0304 Light Chain Variable Domain> (Sequence No. 31)

MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP
GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPLTFGG
GTKVEIKR

The translation starting point of the heavy chain DNA is the ATG codon beginning with adenine (A) at number 55 from the 5′ terminus of Sequence No. 28, and the antibody variable domain extends from the 5′ terminus to the adenine (A) at number 489. In the amino acid sequence, the heavy chain variable domain extends from the N terminus of Sequence No. 29 to the serine (S) residue at number 145. The heavy chain signal sequence was estimated by a gene sequence prediction application (Signal P ver. 2) to extend from the N terminus of Sequence No. 29 to the serine (S) at number 19. The N terminus in the mature form is believed to be the glutamine (Q) at number 20 of Sequence No. 29.

The translation starting point of the light chain DNA is the ATG codon beginning with

the A at number 34 from the 5′ terminus of Sequence No. 30, with the variable domain extending from the 5′ terminus to the adenine (A) at number 414. In the amino acid sequence, the variable domain extends from the N terminus of Sequence No. 31 to the lysine (K) at number 127. The light chain signal sequence was estimated by a gene sequence prediction application (Signal P ver. 2) to extend from the N terminus of Sequence No. 31 to the serine (S) at number 20. The N terminus in the mature form is believed to be the glutamic acid (E) at number 21 of Sequence No. 31.

Table 8 Base Sequences of Synthesized DNA

No.	Primer	Sequence (5′ to 3′)	Length	Seq. No.
1	IgG1	TCTTGTCCACCTTGGTGTTGCTGGGCTTGTG	31-mer	36
2	hk-2	GTTGAAGCTCTTTGTGACGGGCGAGC	26-mer	12
3	hh-4	GGTGCCAGGGGGAAGACCGATGG	23-mer	9
4	hk-5	AGGCACACAACAGAGGCAGTTCCAGATTTC	30-mer	37
5	hk-6	TGGCGGGAAGATGAAGACAGATGGTG	26-mer	13
6	SP6	GATTTAGGTGACACTATAG	19-mer	38
7	T7	TAATACGACTCACTATAGGG	20-mer	39
8	C23LBCL	ATCACAGATCTCTCACCATGGAAGCCCCAGCTCAGCTTCTC	41-mer	40
9	C23Lbsi	GGTGCAGCCACCGTACGTTTGATCTCCACCTTG	33-mer	41
10	T0304Sal	GCGACTAAGTCGACACCATGGACTGGACCTGGAGGATC	38-mer	42
11	T0304Nhe	AGAGAGAGAGGCTAGCTGAGGAGACGGTGACC	32-mer	443
12	SEQU1783	GGTACGTGAACCGTCAGATCGCCTGGA	27-mer	44
13	SEQU4618	TCTATATAAGCAGAGCTGGGTACGTCC	27-mer	45

(3) cDNA Cloning of KMTR1 Antibody Genes

Hybridoma KMTR1 cells were collected by means of centrifugation, and about 900 μg of RNA were purified in accordance with protocol using the RNA extraction reagent ISOGEN (Nippon Gene). Next, 13 μg of polyA$^+$ RNA were obtained from 300 μg of the RNA using Oligotex™-dT30 <Super> (Takara Shuzo). The resulting polyA$^+$ RNA was used as the material in a SMART RACE cDNA Amplification Kit (Clontech Company), and a cloning experiment was performed in accordance with the attached instruction manual to obtain cDNA from an antibody gene variable domain. Specifically, first strand cDNA synthesis was performed by means of a reverse transcriptase with 1.0 μg of purified polyA$^+$ RNA as the raw material. The resulting cDNA was used as the template to amplify the H chain leader sequence and variable domain (hereinafter referred to also as "HV") and the L chain leader sequence and variable

domain (hereinafter referred to also as "LV") by means of PCR using PCR primers specific to the DNA of heavy chain (hereinafter referred to also as "H chain") constant domain and light chain (hereinafter referred to also as "L chain") constant domain of human antibodies (IgGlp for the H chain, hk-2 for the L chain), and UMP primers provided with the SMART RACE cDNA Amplification Kit (oligonucleotides complementary to the common sequences formed on the 5′ terminus of synthesized cDNA) as the primer set. For the PCR, TaKaRa LA Taq™ (Takara Shuzo), which is a Taq DNA polymerase for LA PCR, was used. The template DNA were added to a solution containing 400 μM (final concentration) each of 1 × LA PCR Buffer II (Mg^{2+} plus) and dNTP Mixture, 0.2 μM of two types of primer and TaKaRa LA Taq 2.5 U/50 μl, and reacted by touchdown PCR (5 seconds at 94 °C and 3 minutes at 72 °C (5 cycles), 5 seconds at 94 °C, 10 seconds at 70 °C and 3 minutes at 72 °C (5 cycles), 5 seconds at 94 °C, 10 seconds at 68 °C, and 3 minutes at 72 °C (20 cycles)). After collecting the amplified PCR fragments by ethanol precipitation, they were recovered by agarose gel electrophoresis, then purified in a QIAquick Gel Extraction Kit (Qiagen) which is a DNA purifying kit. The DNA base sequences of the purified HV and LV fragments were determined used ABI PRISM® 3700 DNA Analyzer (Applied Biosystems). Additionally, the amplified HV and LV fragments were subcloned with pGEM®-T Easy Vector System (Promega) using a TA cloning method, and the base sequences of the inserted DNA in the plasmid DNA of the resulting clones were analyzed and compared with the direct sequence analysis results for the PCR product. The primer sequences used to determine the DNA base sequences (hh-4 for the H chain, hk-5 and hk-6 fo the L chain and SP6 and T7 for pGEM®-T Easy Vector) are shown in Table 8. The direct sequence analysis results for the PCR fragments of the HV and LV completely matched the DNA base sequence analysis results of a plurality of clones which were subcloned. The DNA base sequences and amino acid sequences as determined for the H chain and L chain of human antibody genes expressed in the KMTR1 cells are as follows.

<KMTR1 Heavy Chain Variable Domain> (Sequence No. 32)

GAGCTCTGAG AGAGGAGCCC AGCCCTGGGA TTTTCAGGTG TTTTCATTTG GTGATCAGGA
CTGAACAGAG AGAACTCACC ATGGAGTTTG GGCTGAGCTG GCTTTTTCTT GTGGCTATTT
TAAAAGGTGT CCAGTGTGAG GTACAGCTGT TGGAGTCTGG GGGAGGCTTG GTACAGCCTG
GGAGGTCCCT GAGACTCTCC TGTGCAGCCT CTGGATTCAC CTTTAGCAGC TATGCCATGA
GCTGGGTCCG CCAGGCTCCA GGGAAGGGGC TGGAGTGGGT CTCAGCTATT AGTGGTAGTG
GTGGTAGCAG ATACTACGCA GACTCCGTGA AGGGCCGGTT CACCATCTCC AGAGACAATT
CCAAGAACAC GCTGTATCTG CAAATGAACA GCCTGAGAGC CGAGGACACG GCCGTATATT
ACTGTGCGAA AGAGAGCAGT GGCTGGTTCG GGGCCTTTGA CTACTGGGGC CAGGGAACCC
TGGTCACCGT CTCCTCA

<KMTR1 Heavy Chain Variable Domain> (Sequence No. 33)

MEFGLSWLFL VAILKGVQCE VQLLESGGGL VQPGRSLRLS CAASGFTFSS YAMSWVRQAP
GKGLEWVSAI SGSGGSRYYA DSVKGRFTIS RDNSKNTLYL QMNSLRAEDT AVYYCAKESS
GWFGAFDYWG QGTLVTVSS

<KMTR1 Light Chain Variable Domain> (Sequence No. 33)

GATCTTAAAA GAGGTTCTTT CTCTGGGATG TGGCATGAGC AAAACTGACA AGTCAAGGCA
GGAAGATGTC GCCATCACAA CTCATTGGGT TTCTGCTGCT CTGGGTTCCA GCCTCCAGGG
GTGAAATTGT GCTGACTCAG TCTCCAGACT TTCAGTCTGT GACTCCAAAG GAGAAAGTCA
CCATCACCTG CCGGGCCAGT CAGAGCATTG GTAGTAGCTT ACACTGGTAC CAGCAGAAAC
CAGATCAGTC TCCAAAGCTC CTCATCAAGT ATGCTTCCCA GTCCTTCTCA GGGGTCCCCT
CGAGGTTCAG TGGCAGTGGA TCTGGGACAG ATTTCACCCT CACCATCAAT AGCCTGGAAG
CTGAAGATGC TGCAGCGTAT TACTGTCATC AGAGTAGTAG TTTACCGATC ACCTTCGGCC
AAGGGACACG ACTGGAGATT AAACGA

<KMTR1 Light Chain Variable Domain> (Sequence No. 33)

MSPSQLIGFL LLWVPASRGE IVLTQSPDFQ SVTPKEKVTI TCRASQSIGS SLHWYQQKPD QSPKLLIKYA SQSFSGVPSR FSGSGSGTDF TLTINSLEAE DAAAYYCHQS SSLPITFGQG TRLEIKR

The translation starting point of the heavy chain DNA is the ATG codon beginning with adenine (A) at number 81 from the 5′ terminus of Sequence No. 32, and the antibody variable domain extends from the 5′ terminus to the adenine (A) at number 497. In the amino acid sequence, the heavy chain variable domain extends from the N terminus of Sequence No. 33 to the serine (S) residue at number 139. The heavy chain signal sequence was estimated by a gene sequence prediction application (Signal P ver. 2) to extend from the N terminus of Sequence No. 33 to the serine (S) at number 19. The N terminus in the mature form is believed to be the glutamic acid (E) at number 20 of Sequence No. 33.

The translation starting point of the light chain DNA is the ATG codon beginning with the A at number 66 from the 5′ terminus of Sequence No. 34, with the variable domain extending from the 5′ terminus to the adenine (A) at number 443. In the amino acid sequence, the variable domain extends from the N terminus of Sequence No. 35 to the lysine (K) at number 126. The light chain signal sequence was estimated by a gene sequence prediction application (Signal P ver. 2) to extend from the N terminus of Sequence No. 35 to the serine (S) at number 19. The N terminus in the mature form is believed to be the glutamic acid (E) at number 20 of Sequence No. 30.

Example 15 Production of Recombinant Antibodies

The recombinant antibody expression vectors constructed in Example 14 were introduced into host cells to form recombinant antibody expressing cells. As the host cell for expression, for example, a dhfr-missing line of CHO cells (ATCC CRL-9096) was used. The vectors were introduced into the host cells by electroporation. About 2 μg of the antib0dy expressing vector were linearized by restriction enzymes, after which the genes were introduced into 4×10^6 CHO cells under conditions of 350 V and 500 μF using a Bio-Rad electrophoreter, and inoculated onto a 96-well culture plate. A chemical agent corresponding

to an expression vector selection marker was added and cultivation was continued. After colonies could be discerned, antibody-expressing strains were selected out by the method of Example 4. The purification of antibodies from the selected cells was performed in accordance with Example 10.

Example 16 Apoptosis-Inducing Activity of Recombinant Antibodies in Cancer Cells

The recombinant human anti-TRAIL-R2 monoclonal antibodies obtained in Example 15 were used to measure the apoptosis-inducing activity with respect to the colon cancer cell Colo205 (ATCC No. CCL-222). Colo205 cells cultivated in an RPMI culture medium containing 10% FCS were adjusted to a concentration of 1.0×10^5/ml, and 100 μl were injected into each well of a 96-hole flat-bottom plate (Becton Dickinson). After cultivation for 24 hours at 37 °C in the presence of carbon dioxide gas, 10 μl per well of the purified antibodies E11 (CHO-3) and H48 (CHO-8) were added to a final concentration of 10, 100, 1000 or 10000 ng/ml, and 10 μl of caprine anti-human IgG(γ)-specific polyclonal antibody (Sigma) were added to each well so as to make the final concentration 10 or 100 μg/ml. Some wells in which caprine anti-human IgG(γ)-specific polyclonal antibody was not added were also prepared for the obtained hybridomas. As a positive control, human recombinant TRAIL protein (R&D Systems) was used at final concentrations of 1 and 10 ng/ml. Human anti-HAS antibody was used as a negative control. After cultivation for 48 hours at 37 °C in the presence of 5.0% carbon dioxide gas, an MTS reagent (Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega) was prepared in accordance with the method given in the instruction manual, and 20 μl were added to each well. After cultivating for another 2 hours at 37 °C in the presence of 5.0% carbon dioxide gas, the absorption at a wavelength of 490 nm (reference wavelength 630 nm) was measured using a microplate reader (1420 ARVO multilabel counter, Wallac), and the cell survival rate was computed using the mitochondrial reduction rate as an indicator.

The results are shown in Figs. 11a and 11b. The results of an experiment in which caprine anti-human IgG(γ)-specific polyclonal antibody was not added are shown in Fig. 11a, and the results of an experiment in which caprine anti-human IgG(γ)-specific polyclonal antibody was added are shown in Fig. 11b.

Fig. 11a clearly shows that the recombinant antibodies E11 (CHO-3) and H48 (CHO-3) have apoptosis-inducing activity in Colo205 cells even when the antibodies are used alone.

Additionally, Fig. 11b clearly shows that the same level of apoptosis-inducing activity as in antibodies purified from the conditioned media of the hybridomas is exhibited when caprine anti-human IgG(γ)-specific polyclonal antibody is added.

All publications, patents and patent applications cited in this specification are hereby incorporated into the present specification by reference.

INDUSTRIAL APPLICABILITY

The present invention offers molecules which are useful as preventive or therapeutic agents for diseases caused by cells expressing TRAIL-R1 and R2, especially as drugs for treating malignant tumors, capable of avoiding damage to the liver and with an extremely high level of safety.

SEQUENCE TABLE FREE TEXT

Sequence No. 1: synthesized DNA

Sequence No. 2: synthesized DNA

Sequence No. 3: synthesized DNA

Sequence No. 4: synthesized DNA

Sequence No. 5: synthesized DNA

Sequence No. 6: synthesized DNA

Sequence No. 7: synthesized DNA

Sequence No. 8: synthesized DNA

Sequence No. 9: synthesized DNA

Sequence No. 10: synthesized DNA

Sequence No. 11: synthesized DNA

Sequence No. 12: synthesized DNA

Sequence No. 13: synthesized DNA

Sequence No. 14: synthesized DNA

Sequence No. 15: synthesized DNA

Sequence No. 36: synthesized DNA

Sequence No. 37: synthesized DNA

Sequence No. 38: synthesized DNA

Sequence No. 39: synthesized DNA

Sequence No. 40: synthesized DNA

Sequence No. 41: synthesized DNA

Sequence No. 42: synthesized DNA

Sequence No. 43: synthesized DNA

Sequence No. 44: synthesized DNA

Sequence No. 45: synthesized DNA

CLAIMS

1. An antibody binding to TRAIL-R1 and/or TRAIL-R2 or a functional fragment thereof.

2. An antibody or functional fragment thereof as recited in claim 1, having at least one selected from among the following properties (a)-(c):

(a) having activity for inducing apoptosis in cancer cells expressing TRAIL-R1 and/or TRAIL-R2;

(b) not affecting healthy human cells expressing TRAIL-R1 and/or TRAIL-R2; and

(c) not causing damage to human hepatocytes.

3. An antibody or functional fragment thereof having all of the following properties (a)-(c):

(a) having activity for inducing apoptosis in cancer cells expressing TRAIL-R1 and/or TRAIL-R2;

(b) not affecting healthy human cells expressing TRAIL-R1 and/or TRAIL-R2; and

(c) not causing damage to human hepatocytes.

4. An antibody or functional fragment thereof as recited in claim 2 or 3, binding to TRAIL-R2 but not binding to TRAIL-R1.

5. An antibody or functional fragment thereof as recited in claim 2 or 3, binding to TRAIL-R2 and also binding to TRAIL-R1.

6. An antibody or functional fragment thereof as recited in any one of claims 1-5, characterized by being a monoclonal antibody produced by a mouse-mouse hybridoma.

7. An antibody or functional fragment thereof as recited in any one of claims 1-6, characterized by being a human antibody.

8. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24

hours is at least 0.01 μg/ml.

9. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is at least 0.1 μg/ml.

10. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is 2-10 μg/ml.

11. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is at least 10 μg/ml.

12. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is 10-100 μg/ml.

13. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is at least 100 μg/ml.

14. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours is at most 100 μg/ml.

15. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours is at most 10 μg/ml.

16. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours is at

most 0.7 μg/ml.

17. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours is 0.02-0.11μg/ml.

18. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours is at most 0.02 μg/ml.

19. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is 2-100 μg/ml, and the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours is 0.02-0.11μg/ml.

20. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is at least twice the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours.

21. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is at least 10 times the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours.

22. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is at least 50 times the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours.

23. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24

hours is 50-100 times the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours.

24. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is at least 100 times the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours.

25. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is 100-1000 times the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours.

26. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is 250-1000 times the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours.

27. An antibody or functional fragment thereof as recited in any one of claims 1-7, wherein the LD50 value for human hepatocytes with 7.5×10^4 cells and a reaction time of 24 hours is at least 1000 times the LD50 value for cancer cells with 2.5×10^3 cells and a reaction time of 48 hours.

28. An antibody or functional fragment thereof as recited in any one of claims 8-27, wherein the reaction volume is 110-120 μl.

29. An antibody or functional fragment thereof as recited in any one of claims 2, 3 and 14-28, wherein the cancer cells are Colo205 cells.

30. An antibody or functional fragment thereof as recited in either claim 2 or 3, wherein the cancer cells are Colo205 cells, U251 cells or Jurkat cells.

31. An antibody or functional fragment thereof as recited in any one of claims 1-30, capable of inhibiting growth of a tumor or causing regression of a tumor.

32. An antibody or functional fragment thereof as recited in claim 31, wherein the tumor is of at least one type chosen from among colon cancer, colorectal cancer, lung cancer, mammary cancer, brain tumors, melanoma, renal cell cancer, cystic cancer, leukemia, lymphoma, T-cell lymphoma, multiple myeloma, gastric cancer, pancreatic cancer, cervical cancer, endometrial cancer, ovarian cancer, esophagal cancer, hepatic cancer, squamous cancer, skin cancer, urethral cancer, prostate cancer, chorionic cancer, pharyngeal cancer, laryngeal cancer, pleural cancer, androblastoma, endometrial hyperplasia, endometriosis, embryoma, fibrosarcoma, Kaposi sarcoma, hemangioma, cavernous hemangioma, angioblastoma, retinoblastoma, astrocytoma, neurofibroma, oligodendroglioma, medulloblastoma, neuroblastoma, neuroglioma, rhabdomyosarcoma, glioblastoma, osteogenic sarcoma, leiomyosarcoma, goiter and Wilms tumors.

33. An antibody or functional fragment thereof as recited in claim 31, wherein the tumor is from Colo205 cells transplanted into nude mice.

34. An antibody or functional fragment thereof as recited in any one of claims 31-33, wherein the period of time over which growth of a tumor is inhibited or regression of a tumor is caused lasts at least 9 days.

35. An antibody or functional fragment thereof as recited in any one of claims 31-34, wherein the dosage of the antibody or functional fragments is 100 μg/body or 5 mg/kg.

36. An antibody or functional fragment thereof as recited in any one of claims 31-34, wherein the dosage of the antibody or functional fragments is 20 μg/body or 1 mg/kg.

37. An antibody or functional fragment thereof as recited in any one of claims 31-34, wherein the dosage of the antibody or functional fragments is 4 μg/body or 200 μg/kg.

38. An antibody or functional fragment thereof as recited in any one of claims 31-34,

wherein the dosage of the antibody or functional fragments is 1 μg/body or 50 μg/kg.

39. An antibody or functional fragment thereof as recited in any one of claims 1-38, characterized by being an immunoglobulin G type antibody.

40. An antibody or functional fragment thereof, capable of causing shrinkage of a tumor of on average at least 14% four days after an initial treatment, when administered at a concentration of 20 μg/mouse to 4-6 week old cancerous mice having tumors of 100 mm^3.

41. An antibody or functional fragment thereof as recited in claim 40, wherein the shrinkage of the tumor of on average at least 14% is sustained for at least 7 days.

42. An antibody or functional fragment thereof as recited in claim 40, capable of causing shrinkage of a tumor of on average at least 65% four days after an initial treatment, when administered at a concentration of 20 μg/mouse to 4-6 week old cancerous mice having tumors of 100 mm^3.

43. An antibody or functional fragment thereof as recited in claim 40, capable of causing shrinkage of a tumor of on average at least 80% seven days after an initial treatment, when administered at a concentration of 20 μg/mouse to 4-6 week old cancerous mice having tumors of 100 mm^3.

44. An antibody or functional fragment thereof as recited in claim 43, wherein the shrinkage of the tumor of on average at least 80% is sustained for at least 4 days.

45. An antibody or functional fragment thereof as recited in claim 40, capable of causing shrinkage of a tumor of on average at least 45% three days after an initial treatment, when administered at a concentration of 25 μg/mouse to 12 week old cancerous mice having tumors of 100 mm^3.

46. An antibody or functional fragment thereof as recited in claim 45, capable of causing shrinkage of a tumor of on average at least 65% five days after an initial treatment, when

administered at a concentration of 25 μg/mouse to 12 week old cancerous mice having tumors of 100 mm^3.

47. An antibody or functional fragment thereof as recited in claim 46, wherein the shrinkage of the tumor of on average at least 65% is sustained for at least 27 days.

48. An antibody or functional fragment thereof as recited in claim 40, capable of causing shrinkage of a tumor of on average at least 39% four days after an initial treatment, when administered at a concentration of 20 μg/mouse to 4-6 week old cancerous mice having tumors of 300 mm^3.

49. An antibody or functional fragment thereof as recited in claim 48, wherein the shrinkage of the tumor of on average at least 39% is sustained for at least 14 days.

50. An antibody or functional fragment thereof as recited in claim 40, characterized by being 0304 antibody.

51. An antibody or functional fragment thereof as recited in claim 40, characterized by being E-11-13 antibody.

52. An antibody or functional fragment thereof capable of binding to TRAIL-R1 and/or TRAIL-R2, produced by hybridoma E-11-13, H-48-2, L-30-10, N-18-12, W-40-5, X-14-4, X-51-12, F-4-8, G-3-10, 0304 or KMTR1.

53. An antibody or functional fragment thereof capable of binding to TRAIL-R1 and/or TRAIL-R2, produced by hybridoma H-48-2 of Deposit No. FERM BP-7599, hybridoma E-11-13 of Deposit No. FERM BP-7698 or FERM BP-7770, hybridoma F-4-8 of Deposit No. FERM BP-7699 or FERM BP-7768, hybridoma L-30-10 of Deposit No. FERM BP-7700 or FERM BP-7769, hybridoma 0304 of Deposit No. FERM BP-8037 or hybridoma KMTR1 of Deposit No. FERM BP-8038.

54. An antibody or functional fragment thereof, having an amino acid sequence at a

mature portion comprising the heavy chain variable domain and light chain variable domain of an antibody produced by hybridoma E-11-13 as respectively indicated by Sequences Nos. 17 and 19, the heavy chain variable domain and light chain variable domain of an antibody produced by hybridoma L-30-10 as respectively indicated by Sequences Nos. 21 and 23, the heavy chain variable domain and light chain variable domain of an antibody produced by hybridoma H-48-2 as respectively indicated by Sequences Nos. 25 and 27, the heavy chain variable domain and light chain variable domain of an antibody produced by hybridoma 0304 as respectively indicated by Sequences Nos. 29 and 31, and the heavy chain variable domain and light chain variable domain of an antibody produced by hybridoma KMTR1 as respectively indicated by Sequences Nos. 33 and 35.

55. An antibody or functional fragment thereof having an amino acid sequence at a mature portion comprising the heavy chain variable domain and light chain variable domain coded by a nucleic acid sequence isolated from hybridoma E-11-13 as respectively indicated by Sequences Nos. 16 and 18, the heavy chain variable domain and light chain variable domain coded by a nucleic acid sequence isolated from hybridoma L-30-10 as respectively indicated by Sequences Nos. 20 and 22, the heavy chain variable domain and light chain variable domain coded by a nucleic acid sequence isolated from hybridoma H-48-2 as respectively indicated by Sequences Nos. 24 and 26, the heavy chain variable domain and light chain variable domain coded by a nucleic acid sequence isolated from hybridoma 0304 as respectively indicated by Sequences Nos. 28 and 30, and the heavy chain variable domain and light chain variable domain coded by a nucleic acid sequence isolated from hybridoma KMTR1 as respectively indicated by Sequences Nos. 32 and 34.

56. A hybridoma producing a monoclonal antibody binding to TRAIL-R2, chosen from the group consisting of E-11-13, H-48-2, L-30-10, N-18-12, W-40-5, X-14-4, X-51-12, F-4-8, G-3-10, 0304 and KMTR1.

57. A hybridoma producing a monoclonal antibody binding to TRAIL-R2, chosen from the group consisting of by hybridoma H-48-2 of Deposit No. FERM BP-7599, hybridoma E-11-13 of Deposit No. FERM BP-7698 or FERM BP-7770, hybridoma F-4-8 of Deposit No. FERM BP-7699 or FERM BP-7768, hybridoma L-30-10 of Deposit No. FERM BP-7700 or FERM BP-7769,

hybridoma 0304 of Deposit No. FERM BP-8037 or hybridoma KMTR1 of Deposit No. FERM BP-8038.

58. A method for producing an anti-TRAIL-R2 monoclonal antibody, characterized by cultivating a hybridoma as recited in claim 56 or 57; and collecting antibodies binding to TRAIl-R2 from the resulting culture.

59. A method for producing an anti-TRAIL-R2 monoclonal antibody, characterized by isolating a gene coding for an anti-TRAIL-R2 monoclonal antibody from a hybridoma as recited in claim 56 or 57; constructing an expression vector having said gene; introducing said expression vector into a host and causing expression of said monoclonal antibody; and collecting the anti-TRAIL-R2 monoclonal antibody from the resulting host, conditioned medium of the host or secretions from the host.

60. A method as recited in claim 59, wherein the host is chosen from the group consisting of *E. coli*, yeast cells, insect cells, mammalian cells and vegetable cells as well as mammals.

61. A preventive or therapeutic agent for tumors, containing an antibody or functional fragment thereof as recited in any one of claims 1-55 as an active ingredient.

62. A preventive or therapeutic agent for tumors as recited in claim 61, wherein the tumors are of at least one type chosen from among colon cancer, colorectal cancer, lung cancer, mammary cancer, brain tumors, melanoma, renal cell cancer, cystic cancer, leukemia, lymphoma, T-cell lymphoma, multiple myeloma, gastric cancer, pancreatic cancer, cervical cancer, endometrial cancer, ovarian cancer, esophagal cancer, hepatic cancer, squamous cancer, skin cancer, urethral cancer, prostate cancer, chorionic cancer, pharyngeal cancer, laryngeal cancer, pleural cancer, androblastoma, endometrial hyperplasia, endometriosis, embryoma, fibrosarcoma, Kaposi sarcoma, hemangioma, cavernous hemangioma, angioblastoma, retinoblastoma, astrocytoma, neurofibroma, oligodendroglioma, medulloblastoma, neuroblastoma, neuroglioma, rhabdomyosarcoma, glioblastoma, osteogenic sarcoma, leiomyosarcoma, goiter and Wilms tumors.